LXXXIV. PHENOL METHYL ETHERS AS OESTROGENIC AGENTS

By BERNHARD ZONDEK and ERNST BERGMANN

From the Laboratory of the Gynecological and Obstetrical Department of the Rothschild Hadassah Hospital, Jerusalem, and the Daniel Sieff Research Institute, Rehovoth, Palestine

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The experimental material presented in this paper derives from two sources: (*a*) the observation that oestrone methyl ether is a powerful oestrogenic agent, not much less active than oestrone itself; (*b*) the occasional finding that fennel oil (*oleum foeniculi*) has a remarkable oestrogenic potency (500 rat units per ml. tested on infantile rats).

(*a*) Conventionally, the oestrogenic activity of oestradiol benzoate is ascribed to its hydrolytic fission in the body, the real agent being assumed to be the free phenolic compound [Miescher & Scholz, 1937]. This assumption is somewhat invalidated by the fact that oestrone methyl ether and oestradiol methyl ether produce the full vaginal cycle even in doses of 1–5γ (under conditions where 0·5γ of oestrone is active) although no demethylation would take place in the cell. Apparently, the free hydroxyl group is not at all essential for the activity of the molecule; its particular "haptophoric" properties are not required.

(*b*) Fennel oil is not the first product of the vegetable kingdom to exhibit oestrogenic activity. Oestrogenic activity has been found in flowers, potatoes, beetroot, yeast, by various authors as early as 1927 and 1929 [Loewe *et al.* 1927; Faure *et al.* 1927; Dingemanse & Laqueur, 1929]. Furthermore, it has been stated [Butenandt & Jacobi, 1933] that palm kernel oil produces the vaginal cycle in castrated mice, and the same holds true according to Aschheim & Hohlweg [1933] for petroleum and peat. But their activities are of quite a different magnitude from that of fennel oil (petroleum, peat and coal contain about 4000–2000 M.U./kg., the plant products mentioned even less, fennel oil 500,000 infantile R.U./kg.). A second main difference is that the activity of the above-named vegetable products is due to the presence of oestrone which has actually been isolated in crystalline state from palm kernel oil, while this possibility is excluded for fennel oil, which is prepared by steam-distillation. The action of the latter has undoubtedly to be ascribed to other "unspecific" oestrogenic agents.

In extending these experiments, a series of other natural essential oils has been tested. The results are reported in Table I. It is noteworthy that both the oils (fennel and anise oils) which showed marked oestrogenic activities derive from closely related plant species (Umbelliferae). On the other hand, the inactive dill oil derives also from an Umbellifera.

We were therefore led to the assumption that the activity is due to certain specific constituents, and started an investigation as to the nature of these constituents. Amongst them, anethole, which is present in both fennel and anise oils,

proved to be fairly active, and as it is related to oestrone methyl ether and oestradiol methyl ether, experiments were conducted to include various phenolic

Table I. *Oestrogenic activity of essential oils*

Name of the oil	Activity per ml.	Main constituents of the oil [Wehmer, 1929, 1931]
Fennel oil (*oleum foeniculi*)	500 R.U. (<50 M.U.)	Anethole, *p*-methoxyphenylacetone, *d*-fenchone, etc.
Anise oil (*oleum anisi*)	100 R.U. (<10 M.U.)	Anethole, estragol
Muscat oil (*oleum macidis*)	—	(+)-Pinene, (+) camphene, eugenole, *iso*eugenol, safrole
Eucalyptus oil	10 R.U. (0 M.U.)	Cineol, (+)-pinene, fenchene, camphene
Dill oil (*Anethum graveolens*)	—	Carvone, phellandrene, apiole
Bay oil (*Pimenta officinalis*)	—	Eugenol
Pine oil (*Pinus silvestris*)	—	Pinene, camphene, fenchone, etc.
Oil from Jaffa orange peel	—	Limonene, *d*-linalool, methyl anthranilate
Thymus oil (*Thymus vulgaris*)	—	Thymol, *p*-cymene, borneol
Oil of cloves (*oleum caryophyllorum*)	— (toxic)	Eugenol, acetyleugenol, caryophyllene, etc.
Oil of cinnamon (*Cinnamomum ceylonium*)	— (toxic)	Cinnamic aldehyde, eugenol, phellandrene

methyl ethers and similar derivatives.[1] We detected a certain activity in some of the tested materials, as may be seen from Table II. Even the most potent agents amongst them are much less active then oestrone itself. Unfortunately, no rule governing the correlation between chemical structure and oestrogenic activity in these series can be derived from Table II. In the *p*-methoxy-series, e.g. the propenyl compound is active, the allyl derivative not, while for the 3:4-dimethoxy-series the reverse holds true.

Table II. *Oestrogenic activity of phenolic methyl ethers*

Name	Activity
Anethole	50 M.U., 100 R.U./ml.
Estragol	—
Dihydroanethole	<10 M.U., 10 R.U./ml.
Anisole	10 M.U./ml. (toxic)
Eugenol methyl ether	— (toxic in 0·1 g. doses)
*iso*Eugenol methyl ether	— (toxic in 0·1 g. doses)
Dihydroeugenol methyl ether	—
Veratrole	—
Anisaldehyde	—
Veratric aldehyde	10 R.U./ml., negative with mice
Vanillin	—
Piperonal	—
p-Methoxycinnamic aldehyde*	— (toxic in 0·1 g. doses)
Veratryl alcohol	— (toxic)
p-Methoxyphenylacetone†	—

* Cinnamic aldehyde and cinnamyl alcohol were also negative.
† Constituent of fennel oil.

It may be noted that hydrogenation of the carbonyl group in veratric aldehyde destroys the activity, while the same operation in the oestrone and androsterone series increases the activity [David *et al.* 1933; 1935; Schoeller *et al.* 1935; Doisy *et al.* 1933]. One obtains the impression from the above data and

[1] As fennel oil is more active than anethole, its oestrogenic activity cannot be ascribed exclusively to that substance, but rather to another still unknown constituent of the natural oil.

from those presented by previous authors [Dodds, 1936, 1, 2], that the activity of a given organic molecule is not determined directly by constitution but by certain other factors, which may or may not be connected with structural features.

It has been pointed out already that the magnitude of activity is much smaller for the substances studied than for oestrone. It was unexpected, therefore, when Dodds & Lawson [1937, 1] reported anol (*p*-propenylphenol), the mother-substance of anethole, to have the same degree of oestrogenic activity as oestrone. We too were unable to confirm these results, which have been recently withdrawn by the British authors [Dodds & Lawson, 1937, 2]. Pure anol is only slightly active (100 M.U., 1000 R.U./g.), but when its preparation from anethole has not been carried out with special care, it is contaminated by a non-crystallizable substance which increases the total activity (1000 M.U., 5000–10,000 R.U./g.), but still many times smaller than that of follicular hormone. Dodds & Lawson tentatively ascribe the formation of this active by-product to a polymerization reaction.[2]

Finally it has to be reported that our experiments showed a different behaviour towards the tested preparations of infantile female rats and castrated mice, respectively. The relevant results are included in Table II. (Similar observations have been reported by Behrens *et al.* [1937] and by Rowe & Simonds [1937].

Experimental

Anethole, anisole, eugenol methyl ether, veratrole, anisaldehyde, vanillin and piperonal were commercial products which we purified by careful distillation or recrystallization; *p*-methoxycinnamic aldehyde was prepared according to Scholtz & Wiedemann [1903]. Estragol was prepared according to Tiffeneau [1904].

Dihydroanethole. Anethole (14·8 g.) in alcoholic solution (300 ml.) absorbed in presence of palladized $BaSO_4$ (1 g., 5%) the theoretical amount of hydrogen (2519 ml., 759 mm.; 34°) within 55 min. at room temperature. The product had B.P. 119°/42 mm. and was obtained in quantitative yield (cp. Albright [1914]).

iso*Eugenol methyl ether.* Eugenol methyl ether (10 g.) was boiled with 7·5% methyl-alcoholic KOH (40 ml.) for 2 hr. The methyl alcohol was evaporated from a water bath as far as possible and the residue taken up with water and ether. B.P. 152°/45 mm.; yield, 7 g. [Eijkman, 1890].

Dihydroeugenol methyl ether. Eugenol methyl ether (8·9 g.) was hydrogenated as above in alcoholic solution (25 ml.). The required amount of hydrogen (1250 ml.; 29°, 756 mm.) was absorbed quickly; the hydrogenated product had B.P. 151–153°/46 mm.[1]

Veratric aldehyde was prepared according to "Organic Syntheses" (vol. **13**, p. 102 (1933); **16**, 91 (1936)) and was converted into *veratryl alcohol* according to Decker & Pschorr [1904]. The aldehyde (46 g.) was boiled for 1 hr. with 15% methyl-alcoholic KOH (140 ml.). The mass was poured out into water (1 l.) and the veratryl alcohol extracted from the alkaline mixture by means of ether. B.P. 125–130°/0·2 mm.; yield, 13 g.

p-*Methoxyphenylacetone.* Estragol (10 g.) in alcohol (150 ml.) was treated with mercuric oxide (45 g.) and solid iodine (51 g.) added in small portions, so as

[1] Catalytic hydrogenation has not been applied before to eugenol methyl ether. Cp. Beilstein, vol. **6**, p. 920 (Berlin 1923).

[2] Recently synthetic dimeric anol has been shown to be active [Campbell *et al.* 1938; *cf.* Serini & Steinwerk, 1937].

to avoid too great a rise in temperature. After 48 hr. the mass was poured into a large volume of water and the estragol methoxyiodide,

$$CH_3O.C_6H_4CH_2.\underset{\displaystyle OCH_3}{\underset{|}{CH}}.CH_2I$$

formed [Daufresne, 1907; 1908, 1, 2; Bougault, 1900] extracted with ether. Even in a high vacuum (B.P. 165–170°/1·5 mm.) the product could not be obtained completely iodine-free and colourless; it was converted directly into *p*-methoxyphenylacetone according to Daufresne.[1] The preparation obtained (37 g.) was kept with silver nitrate (24 g. finely ground) in alcohol (250 ml.) at room temperature for 2 hr. and then boiled for 7 hr. The filtered solution was concentrated, the residue taken up with water and ether and the ethereal layer distilled. B.P. 118–120°/0·2 mm.; yield, 17 g.

Anol. Anethole was hydrolysed according to Stoermer & Kahlert [1901] with alcoholic KOH in an autoclave filled with nitrogen (10 atm. pressure at the beginning). The reaction mixture was taken up with water and ether, the alkaline solution acidified and the anol so obtained extracted with ether. It boils at 95°/0·3 mm., and the colourless distillate crystallizes spontaneously. The crystals, after drying on a porous plate, have M.P. 90°.

Oestrone methyl ether was prepared from oestrone (1 g.) with methyl *p*-toluenesulfonate (1·3 g.) and 10% KOH (9 ml.) [Cohen *et al.* 1935; cp. Butenandt & Hildebrandt, 1931; Marrian & Haslewood, 1932]. The precipitate was recrystallized from methanol, M.P. 170°. In the same way oestradiol gave pure *oestradiol methyl ether*: it was obtained first as an oil, which crystallized on trituration with methyl alcohol and could be recrystallized from the same solvent. M.P. 97° [cp. Danielli *et al.* 1933].

Test methods

For the animal tests, the substances were injected in three portions, within 24 hr.; liquids were used in the homogeneous state, solids in oil solution. Twice daily vaginal smears have been taken; only full oestrus has been taken as positive.

The tests have been carried out with (*a*) adult castrated mice, average weight 20 g., and (*b*) infantile non-castrated rats, average weight 25–30 g., occasionally with (*c*) adult castrated rats, average weight 150–200 g. R.U. (rat units) and therefore do not refer to the conventional test carried out on castrated sexually mature rats, but to infantile rats. As pointed out in the theoretical part of this paper, an unexpected observation has been made in these comparative experiments. Although the infantile rat has about the same weight as the adult mouse, some of the tested materials showed higher activities with rats than with mice (more than tenfold). These observations recall the well-known fact, that for oestrone the requirements of an adult castrated rat (100 g.) are 5 times as great as those of an adult castrated mouse (20 g.), while for oestradiol benzoate the requirements of both the test animals are equal, in spite of the difference in weight. Both the methyl ethers of oestrone and oestradiol proved to behave like oestrone, while fennel oil and anise oil are similar to oestradiol benzoate, but the difference is still more conspicuous (10 times more active for the infantile rat than for the mouse). No difference has been observed between castrated and non-castrated rats, as occasionally asserted for oestriol.

[1] In disagreement with this author's description the ketone was obtained directly, instead of the unstable methoxynitrate.

SUMMARY

1. The methyl ethers of oestrone and oestradiol show not much lower oestrogenic activity than the *O*-methyl-free compounds, their free hydroxyl groups apparently being not essential for their action.

2. In contradistinction to a number of other essential oils, fennel and anise oils and to a very slight extent eucalyptus oil too have definite oestrogenic activities.

3. These activities are to be ascribed to anethole present in these oils. A number of other phenol methyl ethers have been tested for oestrogenic activity among which dihydroanethole and veratric aldehyde gave slight positive responses.

4. Pure anol is also slightly active.

5. A surprising difference in behaviour of non-castrated infantile rats and castrated adult mice towards the substances tested has been observed.

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LXXXV. THE NATURE OF HAEMOGLOBIN IN THE RED BLOOD CORPUSCLE

By GORDON ALBERT ADAMS

From the Department of Biochemistry, Faculty of Medicine, University of Western Ontario, London, Canada

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In a previous communication [Adams *et al.* 1934] it was shown that the ultra-violet band at 4100 Å. which is characteristic of haemoglobin and its immediate derivatives is not found in unlaked blood. This observation was made originally on corpuscle suspensions of horse blood, pure horse haemoglobin solutions and a series of derivatives made from purified horse haemoglobin (Hb). The observation has been extended to several species and confirmed in all cases. The blood specimens examined were goose, turtle, rabbit, horse and human.

Since this fundamental difference has been found, numerous attempts have been made to find out the nature of the Hb inside the corpuscle. Obviously, there is a difference between the Hb inside and outside the corpuscle. It may be a chemical or a physical difference due to different spectral conditions presented by a Hb solution and a corpuscle suspension of Hb. There is a possibility that the absence of the great U.V. band from the corpuscle suspensions might be due to the masking effects of the blood serum, the corpuscle wall, material mixed with Hb in the cell or material combined with Hb but removed ether by laking. As to the serum it has been shown by Lewis [1917] that the U.V. absorption spectrum of blood serum has two peaks at 2800 and 2400 Å. respectively. It represents several superimposed curves and does not resemble that of a pure compound, being due chiefly to protein. Furthermore, it was noted that the great U.V. band of Hb exists in laked blood as well as in aqueous solutions of crystalline Hb. The question as to whether the corpuscles may be a factor can be eliminated by the fact that blood laked either by ether or by freezing and thawing and containing the corpuscle "ghosts" still displays the band in the ultra-violet. Removal of the "ghosts" by centrifuging does not effect the absorption band.

In regard to the presence of cell stroma masking the absorption band, it is evident that it is not a factor since the spectrum of the laked blood containing the empty corpuscles does not differ from that of an aqueous solution of oxy-haemoglobin.

Gibbs *et al.* [1931] call attention to the fact that in a system containing suspended matter capable of absorbing light, the total loss of light at any given wavelength is the sum of two factors, that due to selective absorption and that due to scattering. Two solutions of the same substance which contain different amounts of suspended matter yield absorption curves having the same general form but differing in intensity. This is the case using corpuscle suspensions and laked samples containing the "ghosts". In the case of the corpuscle suspension there is more suspended matter and consequently more scattering. The two absorption spectra should be the same in form but not in intensity. It has been shown that there is not only a change in intensity due to scattering but also a change in form as shown by the absence of the main band at 4100 Å. If the scattering due to the suspended corpuscles were responsible for the absence of the main band, then it would follow that the small band at 2750 Å. would be

lacking also. It is a law of optics that the loss of light due to scattering is greater toward the short wavelengths. However, the band at 2750 Å. appears in the corpuscle suspensions although diminished in its intensity compared with the laked blood spectra.

Experimental

In an attempt to get more direct evidence on the effect of scattering, some experiments were planned in which the volume of the corpuscle was altered and the effect on the U.V. spectrum recorded.

In order to change the volume of the corpuscles they were placed in solutions of different osmotic strengths. The normal, 0·9% saline, was regarded as the standard and others referred to it. Several other strengths varying from 0·5% saline to 1·4% were used. The blood cells were allowed to stand in contact with these solutions for 20 min. before the spectrogram was taken. Below 0·7% the experiments failed because of high cell fragility and resulting haemolysis.

The spectrum of the corpuscles in 0·7% saline shows a slight tendency towards a small band in the general region of 4100 Å.; but this is explained on the basis that there is always a slight degree of haemolysis in the hypotonic solutions and the small amount of Hb which "leaks" out is no doubt responsible for the small absorption at 4100 Å. From theoretical considerations a marked increase in absorption was expected. As a matter of fact, there was little increase in the absorption, showing that the increase in the size of the corpuscle had but little effect on the absorption intensity.

On the other hand, in the 1·2% saline the cells decrease in volume to about one-half their original size and consequently the light-scattering effect should be lessened; as a matter of observation the general absorption is slightly less, but here again the difference is not marked, so it may be concluded that the size of the particle is not more than a 2–4% factor in light absorption in the 4100 Å. region.

The next experiment was an attempt to establish artificial conditions which would approach the nature of the red blood cell suspension. This was done by precipitating Hb in a finely divided state, by adding gum arabic to the Hb solution and then sufficient dilute HCl to bring the Hb to its isoelectric point. This gave a very fine suspension of Hb which did not settle out on standing and was suitable for spectrographic analysis. The spectrum of this preparation was compared with that of a 10% Hb solution. The two spectra are so similar that the effect of precipitation in fine particles does not appear to make any marked difference. Actually the scattering effect of the particles does not amount to more than 5%, an amount which is not regarded as significant in this particular type of work.

Increased Hb concentrations do not affect the nature of the spectrum, which seems to be the same over the range 0·5–10%.

It is concluded on the basis of these experiments that the physical state of the Hb in the corpuscle is not sufficient to explain the lack of the characteristic U.V. band at 4100–4200 Å. It probably does not account for more than 5% of the difference in the absorption.

Since it may be concluded that physical differences between Hb inside and outside the corpuscle are inadequate to account for the lack of the characteristic band when Hb is in the corpuscle, attempts were made to find possible chemical combinations between Hb and cell constituents which would have spectra similar to Hb in the corpuscle. The fact that ether laking produces such marked changes points to the possibility that the cell constituents might be lipoid in nature. However, the fact that laking brought about by the physical processes of freezing and thawing, which do not remove any of the constituents, still gives

the same spectrum as the ether-laked blood indicates that the presence of the band is independent of any ether-soluble substance which is removed by the laking process. It does not exclude the possibility that laking breaks down a lipoid-Hb combination which lacks a characteristic band at 4100 Å.

Very little is known concerning the nature of Hb in the cell. It is not even known definitely whether it exists as a simple solution in a protein envelope or in a loose chemical union with stroma or some other cell constituent. No direct evidence has been brought to show that the Hb is combined with either the stroma protein, lipoid fraction or any other cellular constituents.

In view of the lack of data in this regard attempts were made to combine Hb with some of the corpuscle constituents. The first one used was stroma protein or "stromatin". The stromatin was prepared by the method of Jorpes [1932] with some modification. The first preparations which were made contained approximately 10% Hb as determined by the iron content. The spectrogram of this substance shows a relatively flat curve in the region of 4100–4200 Å. Since the substance contained 10% Hb and yet showed no absorption in the 4100–4200 Å. region, it suggested a combination between the stromatin and Hb. The stromatin was rendered lipoid-free by extraction three times with a 1 : 1 ether-alcohol mixture. The spectrum was again taken, and did not vary essentially from the previous one, showing that the lipoid fraction is not concerned in the spectrogram.

Pure samples of stromatin were prepared, the only impurity encountered being methaemoglobin. Its presence could be quite easily detected spectroscopically. The stromatin was also rendered lipoid-free by alcohol-ether extraction. The U.V. spectrum of this pure stromatin was used as standard for subsequent experiments. The material was prepared for spectrographic examination as follows.

To 2 ml. of the pure stromatin in alkaline solution (100 mg. in 25 ml. *N*/10 NaOH) there were added 10 ml. of 1% Hb solution. The *p*H of the solution was 9·8. The spectrogram was taken after about 5 min. The spectrogram is a typical haemoglobin one with the specific absorption at 4100–4200 Å. Apparently there is no immediate combination between stromatin and Hb. In the next experiment the same mixture was made, but it was heated at 37° for 30 min. The *p*H of this solution was 10·1. It changed from a clear reddish colour to a slightly opalescent yellowish red solution. The typical Hb band at 4100 Å. disappears completely and the general appearance of the absorption curve is similar to that of stromatin. It appears that heating of stromatin and Hb for even a period of 30 min. causes a combination of the two substances to give a new compound which has a spectrum very similar to that of stromatin. Heating the stomatin and Hb for 1 hr. instead of 30 min. does not affect the spectrum except to cause less general absorption. As a control experiment the effect of heating haemoglobin with the same amount of alkali as used to dissolve the stromatin was tried. This treatment did not affect the spectrum of the Hb.

A solution of stromatin was made up to a *p*H of 10·1 and heated for 1 hr. at 37° in order to find out if heating causes any change in its spectrum. There are no changes in the specific form of the spectrum curve, although the general absorption is slightly greater. Since it was possible to identify in the lipoid fraction extracted from the crude stromatin both cholesterol and lecithin, attempts were made to combine these substances with Hb. This experiment might seem unnecessary since the entire crude lipoid fraction did not show any signs of combination with Hb, but the possibility that the substance in pure form might react differently could not be disregarded.

The method employed was to add a few mg. of pure cholesterol to a drop of olive oil and 1 drop of $N/10$ NaOH. This mixture was then added to 10 ml. of a 0·5% Hb solution, shaken up thoroughly and heated for a period of 30 min. at 37°. At the end of the heating period, the spectrogram was taken. The spectrum curve is typical for Hb showing a distinct absorption band at 4100–4200 Å. Longer

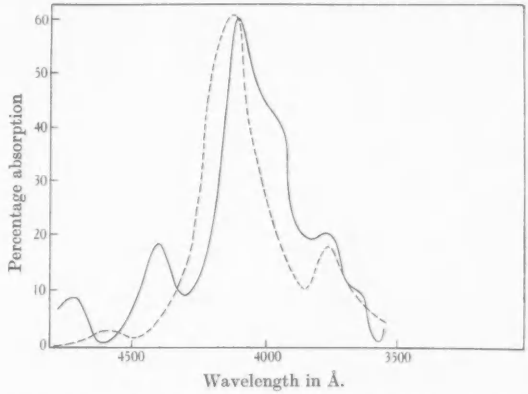

Fig. 1. Haemoglobin in finely precipitated state ——. Haemoglobin in 10% solution – – –.

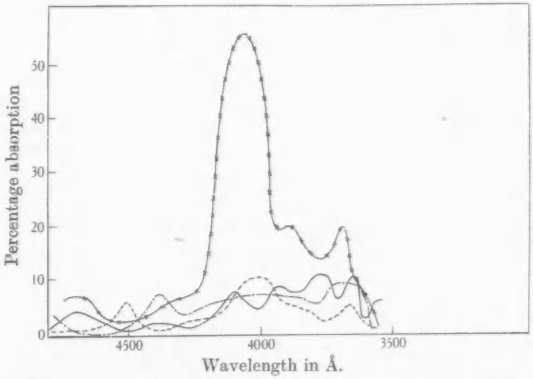

Fig. 2. Stromatin ——. Stromatin (lipoid-free) —·—. Stromatin-haemoglobin (no heating) ×—×. Stromatin-haemoglobin (heated 1 hr.) – – –.

heating periods (up to 3 hr.) gave approximately the same result. The same experiment was tried with lecithin. The results again do not indicate any combination between Hb and lecithin that can be detected spectrographically.

Experimental evidence has been adduced by Litarczek [1933] to show that glutathione added to a Hb solution in the presence of an alkali causes the Hb spectrum bands in the visible region to become decreased or entirely to disappear. Litarczek's method of adding crystalline glutathione to a solution of Hb and heating to 35° was utilized for various periods, usually 30 min., 1 and 1½ hr. The proportions used were 10 mg. glutathione to 20 ml. of 0·5% Hb with a drop

of $N/10$ NaOH added. The spectrum curve does not show any disappearance of the band in the u.v. and appears to be typical for Hb.

Attempts have been made to find out how the stromatin combines with the Hb molecule. When stromatin and alkaline haematin were heated together in the same way as the stromatin and Hb, no combination could be detected by

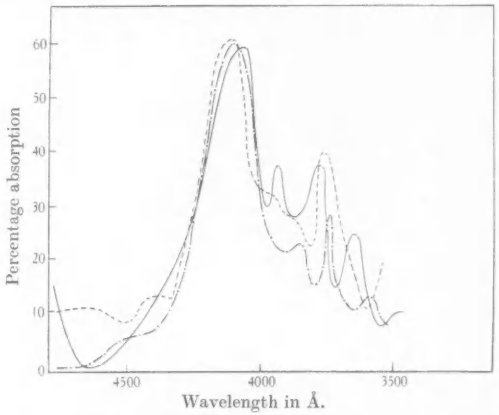

Fig. 3. Haemoglobin + lecithin (heated 1 hr.) —·—. Haemoglobin + cholesterol (heated 1 hr.) – – –. Haemoglobin + glutathione (heated 1 hr.) ———.

changes in the spectrum. Reduced alkaline haematin and stromatin gave a substance with the spectrum of a haemochromogen with the typical 2-banded spectrum in the visible region and a well-defined band in the u.v. at 4000–4100 Å. Alkaline haematoporphyrin and stromatin have likewise shown no signs of combination. The stromatin therefore may be presumed to combine with the globin portion of the Hb molecule.

Isolation of the compound of stromatin and Hb has presented considerable difficulty. The compound appears to be quite labile and is readily broken down by a wide variety of reagents, e.g. many of the common haemolytic agents. Salting out experiments have brought down stromatin and Hb. Isoelectric precipitation has broken down the combination as shown by the presence of the spectrum of Hb.

Summary

Blood corpuscle suspensions do not exhibit an ultra-violet absorption band in the region of 4000–4100 Å. *In vitro* experiments have shown that corpuscle "stromatin" and haemoglobin can be combined to give an ultra-violet spectrum similar to that of haemoglobin in the corpuscle. It is postulated that such a combination exists in the corpuscle.

The compound is a labile one and appears to be a union between stromatin and the globin fraction of the haemoglobin molecule.

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LXXXVI. ESTIMATION OF (CAPON) COMB GROWTH HORMONE IN URINE

By ELISABETH DINGEMANSE and ERNST LAQUEUR

From the Pharmaco-Therapeutic Laboratory of the University of Amsterdam

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Some months ago we published [1937] a method for the determination of capon comb growth-promoting substances ("male hormones") in human urine of males and females of varying ages which involved heating the acidified urine in presence of benzene.

Peterson *et al.* [1937] have also recently published estimations of comb growth-promoting and oestrogenic hormones in human urine after hydrolysis. These authors boiled the urine with 1/10 vol. commercial HCl for $\frac{1}{4}$, 1, 2, 6 or 12 hr., afterwards extracting it according to Gallagher & Koch [1934]. After 2 hr. boiling they found a loss of 40 % of the comb growth-promoting substance as compared with extracts prepared after 15 min. boiling and they therefore assume that comb growth-promoting substances, present in the urine in a combined form, are destroyed by boiling during more than 15 min. in acid medium, or that chlorination occurs. Butenandt *et al.* [1935] describe the formation of chlorodehydroandrosterone from dehydroandrosterone by treatment with alcoholic HCl. Peterson *et al.* [1937] state that neither androsterone nor oestrogens are destroyed by boiling with HCl. We actually find after heating the urine with 150 ml. of 25 % HCl in presence of benzene for two periods of 6 hr. 30–60 I.U. of comb growth-promoting substances per litre, whilst Koch *et al.* find, after boiling for 15 min. with 1/10 vol. HCl in absence of liquid solvents, 64 units, and after 2 hr. boiling under the same conditions only 38 units. The essential difference between the two methods is, that in the American procedure the hormone liberated by acid hydrolysis is exposed for a long time to the acid, whilst in the Amsterdam method the hormone, as soon as it is liberated by acid, passes into the benzene, and is thus withdrawn from the further influence of the acid.

Experimental

The method of Koch *et al.* was compared with ours in a few experiments on the same sample of urine, using our method of extraction but boiling as described by the American authors. For comparison we also tried the method of hydrolysing by addition of 1/10 vol. of conc. H_2SO_4, thus excluding chlorination.

Table I gives the results obtained in the same sample of urine, taking each time 2 l. and treating according to five different methods.

A. The Amsterdam method [Dingemanse *et al.* 1937].

B. The same as A, but using 1/10 vol. conc. H_2SO_4 instead of 150 ml. of 25 % HCl per litre of urine.

C. After addition of 1/10 vol. of 25 % HCl the urine was boiled for 15 min. as described by Koch *et al.* and then neutralized and extracted with benzene according to our own method.

D. The urine was treated as under C but boiled for 2 hr. instead of 15 min.

E. As under D but with addition of 1/5 vol. 25 % HCl.

Table I

No. of exp.	Units of comb growth-promoting hormone found after treatment according to method				
	A	B	C	D	E
428	50	50	50	35	—
429	55	40	40	35	15–25
431	50	35–40	50	50	35
434	40	40	—	—	25
438	40	—	—	—	20

For assaying the extract, 12–15 capons were used (in one assay 9 capons). The following conclusions may be drawn. Methods A and C give the same results in 2 of the 3 cases. How far treatment with H_2SO_4 has caused a loss of the comb growth-promoting hormone cannot be said with certainty, as the number of experiments is too small. Boiling for 2 hr. seems to destroy less hormone in our hands than in those of Koch *et al.* It is clear that the most serious losses occur after heating with 1/5 vol. of acid for 2 hr. In order to examine whether heating for two periods of 6 hr. in a boiling water bath results invariably in complete extraction of comb growth-promoting hormones, we boiled the urine in the experiment number 431 for a third period of 6 hr. No hormone could be detected however in the third extraction and it may therefore be assumed that two periods of 6 hr. are sufficient.

We next examined the resistance of crystalline dehydroandrosterone and androsterone to the different methods of hydrolysis. A known quantity of crystalline dehydroandrosterone was added to urine previously completely extracted. From this mixture the added dehydroandrosterone was completely recovered by method A. With method C 60 % of the activity was lost. The differences between the extracts obtained by methods A and C are thus due to destruction of free dehydroandrosterone by the acid. In natural urine the combined hormone must be hydrolysed before destruction or chlorination can occur and that this hydrolysis must take a considerable time is indicated by the brief contact with acid which is necessary to destroy the free hormone. Dehydroandrosterone boiled in acidified urine for 2 hr. loses all activity and in aqueous suspension it is also destroyed within 2 hr. by 1/10 vol. 25 % HCl. On the other hand, added crystalline androsterone can be quantitatively recovered from urine by method D as stated already by Koch *et al.*

Boiling, according to method E for 2 hr. with 1/5 vol. 25 % HCl, destroyed more than 70 % of the original activity, owing to chlorination or to acid destruction.

In another series of experiments we examined whether dehydroandrosterone is the first of the hormones to disappear from the urine when boiled with acid. Dehydroandrosterone gives an alcohol-insoluble digitonide and can thereby be separated from androsterone. The method for the isolation of dehydroandrosterone is illustrated by the following. 10 mg. of dehydroandrosterone were dissolved in 5 ml. hot 80 % alcohol and to the boiling solution was added a boiling solution of 50 mg. of digitonin in 12 ml. 80 % alcohol. The mixture was kept in a closed centrifuge tube for 24 hr. at room temperature, centrifuged and the precipitate washed twice with ether at the centrifuge. It was then dissolved in 5 ml. of hot pyridine. To this solution ether was added until precipitation of digitonin was complete; after 2 hr. the fluid was centrifuged and the precipitate washed at the centrifuge. The united ether-pyridine solutions were evaporated, and the residue transferred to oil, with the aid of a little acetone. The acetone

was evaporated and the oily extract assayed for its content of dehydroandrosterone. The above technique was applied to alcoholic extracts, prepared from samples of fresh urine according to methods A, C, D and E, 250 mg. of digitonin dissolved in 14 ml. of hot alcohol being added; the material recovered from the precipitate was assayed for dehydroandrosterone, and that remaining in the filtrate, after removal of digitonin, for androsterone.

Table II

	Found after treatment of the urine according to method			
	A	C	D	E
Dehydroandrosterone (units)	8	6	0	0
Androsterone (I.U.)	35	35	35	25

As standard solutions for the comparison two oil solutions were used containing respectively 275γ of crystalline dehydroandrosterone per ml. and 100γ androsterone per ml. A control experiment indicated that 90 % of dehydroandrosterone added to extracted urine could be recovered by this method.

From Table II it appears that according to our own method this urine contained 8 units of dehydroandrosterone and 35 units of androsterone. The ratio of weight calculated from the number of units of each of these two comb growth-promoting substances found in the urine is $8 \times 275 : 25 \times 100$, indicating that 40 % of the quantity of comb growth-promoting substances is contributed by dehydroandrosterone. This relation between dehydroandrosterone and androsterone is in agreement with the values found by other investigators. Schoeller *et al.* [1935], David *et al.* [1935], and Butenandt *et al.* [1935] give ratios of 1:1. After treating the urine by method C a little less dehydroandrosterone was found; it appears that in boiling for 15 min. with acid a small quantity of dehydroandrosterone has already been decomposed. In determining the total content of comb growth-promoting substances in a mixture of androsterone and dehydroandrosterone it was of course impossible to demonstrate a difference of only a few units. That there always exists a risk that hydrolysis by method C inactivates dehydroandrosterone appears from the experiments already mentioned, where boiling free dehydroandrosterone in presence of 1/10 vol. of 25 % HCl for 15 min. resulted in a significant loss of activity. This is in contrast to our own method where urine, freed from comb growth-promoting substances, to which dehydroandrosterone was added, showed no decrease of activity after boiling for two periods of 6 hr. in presence of benzene. After treating the urine by methods D and E no dehydroandrosterone could be isolated by means of the digitonin method, as shown in Table II. In method E the androsterone is also partly inactivated.

We did not examine further whether the loss of activity, after boiling with HCl, is caused by chlorination or by acid destruction. We have however repeated one of this series of experiments, with 1/10 vol. and with 1/5 vol. of 25 % H_2SO_4, with practically identical results; e.g. after boiling for 2 hr. with 1/10 vol. H_2SO_4 20 units instead of 40 units of comb growth-promoting substance remained in the extract; this indicates that acid destruction is more important.

The question remains whether there are in urine comb growth-promoting substances other than dehydroandrosterone, which are still more sensitive to acids and are even destroyed by method A. This could not be answered by boiling with alkali since no hydrolysis of the ethers of comb growth-promoting substances is thus effected, or by enzymic hydrolysis on account of the possible

formation of compounds with greater or less activity. Heating the neutral urine in an autoclave under high pressure and temperature yielded only partial hydrolysis. There remained only the possibility of examining whether a lower concentration of acid gave the same quantity of comb growth-promoting substances.

We pointed out previously [1937] that addition of 25 ml. 25 % HCl to the urine instead of 150 ml. gave a somewhat smaller yield. We now repeated these experiments by adding to three samples of 2 l. each of the same urine 150, 25 and 10 ml. 25 % HCl per litre, after previous neutralization to litmus. The results of these experiments are shown in Table III.

Table III

No. of exp.	25 % HCl added per l. urine			$KHSO_4$ added per l. urine
	A 150 ml.	F 25 ml.	G 10 ml.	150 g.
436	55 units	55 units	50–55 units	—
438	40 units	40 units	40 units	40 units

In this table also an experiment is included in which $KHSO_4$ was substituted for the acid, thus producing a very low acidity. The yield was equal to those in the comparative experiments A, F and G. By further extraction of G at a higher acid concentration no more comb growth-promoting hormone was found so that it appears from these and earlier experiments that at a much lower acid concentration the extract may contain the same activity. With the use of an acid concentration of 10 ml. of 25 % HCl per litre the inactivation of one or more comb growth-promoting substances is possible but unlikely. We can therefore assume with reasonable certainty that the method published by us for the determination of the comb growth-promoting substances in urine gives the correct value.

We agree with Peterson *et al.* that prolonged boiling of strongly acidified urine destroys dehydroandrosterone; however, strong acid also destroys androsterone. We would point out especially that acid destroys activity only in the absence of benzene; in our previous work where quantitative estimations were combined with acid hydrolysis, the hydrolysis was always done in the presence of benzene.

Summary

1. When urine, after addition of 150 ml. 25 % HCl per litre, is heated for two periods of 6 hr. in presence of benzene no loss of comb growth-promoting substances occurs.

2. If 10 ml. 25 % HCl are added instead of 150 ml., the yield of comb growth-promoting hormones remains the same.

3. When free androsterone and/or dehydroandrosterone is added to urine freed from comb growth-promoting substances beforehand and extracted with benzene in the presence of 150 ml. 25 % HCl per litre on a boiling water bath for two periods of 6 hr. recovery of activity is quantitative.

4. When, on the other hand, these acidified urines containing added free hormones are boiled without benzene a loss of 60 % of activity occurs in 15 min. and more than 80 % in 2 hr.

5. Natural urine boiled with 1/10 vol. 25 % HCl in absence of benzene for 15 min. may lose none of its male hormone activity, whilst, when boiled for

2 hr., some loss occurs. Boiling natural urine with double the quantity of acid always diminishes the content of comb growth-promoting substance.

6. After boiling urine in absence of benzene for 2 hr. with 1/10 vol. 25 % HCl, no dehydroandrosterone could be detected.

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LXXXVII. THE PRODUCTION OF ANTISERA TO PREPARATIONS OF PROLACTIN CONTAINING THE GLYCOTROPIC (ANTI-INSULIN) FACTOR OF THE ANTERIOR PITUITARY GLAND

By F. G. YOUNG

From the National Institute for Medical Research, N.W.3

(*Received 16 February 1938*)

The discovery of "antihormones" by Collip and his colleagues [see Collip, 1934] stimulated great interest in the production of antisera to anterior pituitary hormones, and sera capable of inhibiting the action of pituitary gonadotropic, ketogenic and thyrotropic factors, as well as urinary gonadotropic substances, have been extensively investigated during the last few years.

The discovery that the "anti-insulin" or "glycotropic" factor of the anterior pituitary gland is present in preparations of prolactin as usually obtained [Young, 1936] suggested that an antiserum prepared against prolactin might be of some interest, and the possibility of its production was investigated.

Methods

Preparation and assay of prolactin

Prolactin was obtained from commercial (B.D.H. Ltd.) dried ox anterior lobe tissue by a modification of the Bates-Riddle [1935] method described elsewhere [Young, 1938]. The yields, and activities of the different preparations on the pigeon crop gland are shown in Table I.

When 20 mg. of any of these prolactin preparations were injected daily for 5 days into each of a group of immature guinea-pigs, no hypertrophy of the thyroid gland occurred [method of Rowlands & Parkes, 1934]. These preparations therefore contained no significant amount of thyrotropic hormone,[1] but they all possessed glycotropic activity [method of Young, 1936, as subsequently modified, 1938].

Table I

		Pigeon test*	
Preparation	Yield % dried gland	Amount injected daily, mg.	Crop-gland increase/300 g. pigeon, g.
37C	8·62	3	5·0
50C	16·90	3	3·5
52C	12·77	3	4·5

* Method of Rowlands [1937].

Production of antisera

Rabbits and monkeys were the chief donor animals used, although sera from animals of other species, which had received pituitary injections, were also tested.

[1] By thyrotropic hormone is meant the anterior pituitary substance which induces hypertrophy of the thyroid gland. The identity of this substance with that causing increased secretion of the thyroid gland has not been established.

Adult rabbits, bucks or does, received daily subcutaneous injections of prolactin equivalent to 1 g. of dried gland, i.e. 80–170 mg. of prolactin daily. Young female *Rhesus* monkeys (3–4 kg.) generally received twice this dose. Daily injections were maintained for periods up to 9 months. For injection, the prolactin was dissolved in saline at *p*H 8·0 and Seitz-filtered after the addition of 0·4 % tricresol. 20 ml. of blood were removed every week from the ear vein of each rabbit and from the saphenous vein of each monkey, and the samples from a number of animals pooled before separation of the serum.

Testing of antisera

(*a*) *Anti-prolactin activity.* Prolactin preparation 50 C constituted a standard preparation against which the activity of the antisera was tested. When 1–3 mg. of this preparation were given daily to adult pigeons for 6 days the increase in crop-gland weight was approximately proportional to the amount of prolactin administered (Fig. 1). Doses of prolactin in this range were therefore used in the assessment of activity of the antisera.

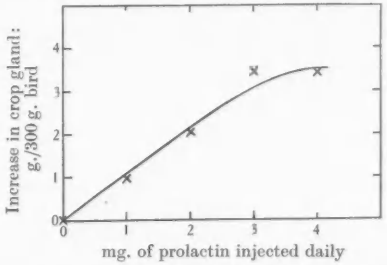

Fig. 1. Dose-response curve for prolactin preparation 50 C. Assays carried out by the method of Rowlands [1937]. Each point represents the average result for a group of at least 10 pigeons.

1 ml. or less of the serum under investigation was injected subcutaneously each day into a pigeon, which simultaneously received a subcutaneous injection of a suitable dose of the standard prolactin into another area of the body. After six such double daily injections the bird was killed and the crop gland weighed. A control test was carried out at the same time using the same dose of prolactin but with normal instead of experimental serum. The average weight of crop gland in a group of 10 birds receiving prolactin together with normal or experimental serum was thus determined. The activity of the experimental serum was indicated by the difference in the average crop gland weight in the two groups. As the average response of a group of our pigeons to a given dose of prolactin is by no means constant from week to week, a control experiment with normal serum was carried out with every test that was made on the experimental sera. For quantitative assay of the inhibitory action of the antiserum it was necessary to adjust the dose of serum to such a level that the response of the crop gland to prolactin was not entirely prevented. If the dose was such that no crop-gland response occurred, the dose of serum used might have been many times that necessary just to prevent the response.

The results are expressed in terms of the weight of crop-gland growth per 300 g. pigeon, which the daily injection of 1 ml. of serum for 6 days could just prevent; e.g. if 0·5 ml. of serum were injected daily the observed difference in crop-gland response of the experimental and control groups was doubled.

(*b*) *Tests with lactating mice.* When antisera were injected daily into lactating mice nursing their young, any depression of milk secretion of the mothers would be recorded as a subnormal growth of the litters. Accordingly, experiments were carried out in which 0·5 or 1 ml. of antiserum was injected subcutaneously into each of a group of lactating mice, the litters being weighed daily. Control experiments were simultaneously carried out using normal serum. The difference in the weight of the young in the two groups was a measure of the ability of the antiserum to inhibit milk secretion in the mothers.

(*c*) *Antiglycotropic activity.* The normal procedure for a test of the glycotropic activity of prolactin is as follows. At 5 p.m. on the day previous to a test, a suitable dose (80–150 mg.) of prolactin is injected subcutaneously into a rabbit and at the same time food is withdrawn. The following morning at 9.30 a.m. the same dose of prolactin is again injected, and at 2 p.m. 2 units of crystalline insulin are administered intravenously, and the blood sugar estimated (Hagedorn-Jensen method) at frequent intervals for 4 hr. afterwards. In a test for antiglycotropic activity of sera, 10 ml. of the antiserum to be tested were administered at the same time as the prolactin in some instances, while in other experiments a number of injections of 5 ml. of antiserum were given during the three days immediately preceding the test. Control experiments with prolactin and normal serum were carried out simultaneously with all these tests on antisera, the dose of prolactin being so adjusted that the hypoglycaemic response to insulin in the control experiments was almost, but not quite, abolished. Antiglycotropic activity of the antiserum was indicated by the extent of the fall of blood sugar in the rabbit which received the experimental serum.

Results

Action of the antisera on the response to prolactin of the pigeon crop gland

The results of experiments with rabbit serum are given in Fig. 2, while those with monkey serum are given in Fig. 3. In view of the great variability of the response of the pigeon crop gland to a given dose of prolactin, the points on these

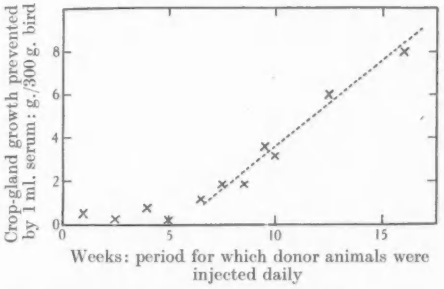

Fig. 2. Rate of appearance of inhibitory activity (pigeon crop-gland test) in the serum of rabbits receiving daily injections of prolactin.

figures fall in a surprisingly regular manner. It will be seen from the curves that the appearance of inhibitory activity is slightly more rapid in rabbits than in monkeys, but that serum with very high activity may be obtained from monkeys which have received daily injections of prolactin for a very long period. The rate of production of inhibitory activity in the monkey appears not to have decreased after $38\frac{1}{2}$ weeks' daily injection of prolactin.

From Fig. 3 it will be seen that sera, obtained after 4, 5 or $6\frac{1}{2}$ weeks of daily prolactin injection, increased rather than decreased the crop-gland response to prolactin in pigeons. The appearance of augmentative action in the serum after 3–7 weeks' daily injection of prolactin has been observed in a number of experiments [Young, 1937], although the augmentation has never been very great. The maximum effect was obtained with the serum of a dog which had received daily injections of prolactin for 5 weeks. In this instance the daily injection of 1 ml. of serum into a pigeon increased the crop-gland response to prolactin by 3·7 g./300 g. bird.

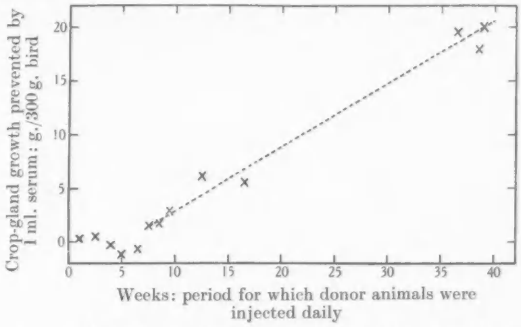

Fig. 3. Rate of appearance of inhibitory activity (pigeon crop-gland test) in the serum of monkeys receiving daily injections of prolactin.

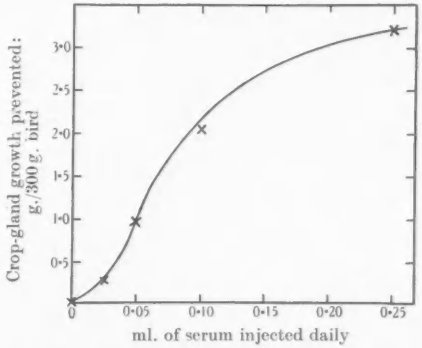

Fig. 4. Curve showing the relationship between the amount of serum injected and the amount of crop-gland growth prevented.

In Fig. 4 is shown the curve obtained from the results of a series of experiments in which different doses of the same monkey antiserum were tested against the same dose of prolactin. The serum used in these experiments was obtained after $38\frac{1}{2}$ weeks of daily prolactin injection.

Action of antiserum on lactating mice

The average weight of the young in the litters of groups of 6 lactating mice receiving daily injections of 0·5 ml. of either rabbit or monkey antiserum was not greatly different from that of a similar control group receiving injections of

normal serum (Fig. 5). For the correct interpretation of this finding, however, it is necessary to take into consideration the fact that many deaths occurred among the young of the mothers which received antiserum, although few young died in the litters of the mothers which received daily injections of normal serum.

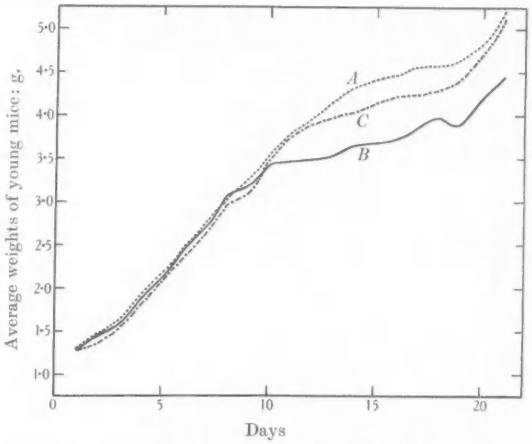

Fig. 5. The average rate of growth of the young of groups of 6 nursing mice which each received daily injections of 0·5 ml. of serum. Curve *A*. Control group receiving normal rabbit serum. Curve *B*. Group receiving rabbit antiserum. Group *C*. Group receiving monkey antiserum.

Thus the average number of young in the litters of the mice receiving rabbit antiserum was 7·7 at the beginning of the experiment, 5·8 after 10 days' injection of antiserum and 3·6 after 21 days. The corresponding figures for the litters whose mothers received monkey antiserum were 7·1, 4·8 and 4·2, and for the control group, receiving normal rabbit serum, 7·5, 6·7 and 6·7.

In a repetition of this type of experiment two daily injections of 0·5 ml. of antiserum (monkey or rabbit) were given to groups of 6 pregnant mice beginning 2–8 days before parturition, and continuing for the first 10 days of the lactation period. Two control groups, receiving either normal rabbit or normal monkey serum, were treated similarly. The results were essentially similar to those for the first part of the previous experiments and therefore need not be given in detail.

The antisera used in these experiments were obtained from rabbits or monkeys which had received daily injections of prolactin for 11–15 weeks.

Influence of antiserum on the glycotropic action of prolactin

After the daily injection of prolactin for 18 weeks, the donor rabbits exhibited a normal hypoglycaemic response to the intravenous injection of 2 units of insulin (Fig. 6). The response to this dose of insulin remained undiminished by the administration of two injections, each of 250 mg., of prolactin, although the initial blood sugar level was somewhat raised. Normal rabbits receiving half this amount of prolactin exhibited practically no hypoglycaemic response to the same dose of insulin (Fig. 6).

It might therefore be expected that the serum of the donor animals would possess the ability to inhibit the glycotropic action of prolactin in normal rabbits. Nevertheless, although indications of a slight antiglycotropic activity of the sera were found in some experiments, the results were so inconsistent that no definite conclusion could be drawn.

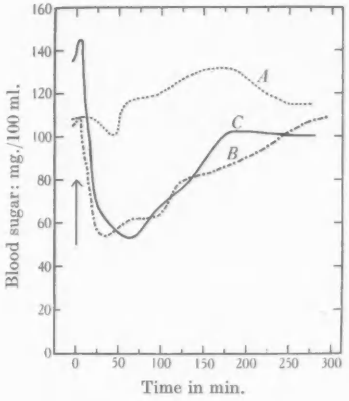

Fig. 6. Blood sugar response in a rabbit receiving 2 units of crystalline insulin, injected intravenously at the time indicated by the arrow. Curve *A*. In a normal rabbit after two injections each of 125 mg. of prolactin. Curve *B*. In a rabbit which had received daily injections of prolactin for 18 weeks. Curve *C*. In a rabbit which had received daily injections of prolactin for 18 weeks and two extra injections of prolactin, each of 250 mg., within the 21 hr. preceding the insulin injection.

Miscellaneous observations

(*a*) *Effect of prolactin injections on pregnant rabbits.* Three pregnant rabbits receiving daily prolactin injections for 5, 11 and 14 days respectively, before parturition, began to secrete milk 2–3 days after the beginning of the period of daily injection, but gave birth to normal healthy litters of 7–10 young. The results of de Fremery [1936], in which abortion or death of the foetus *in utero* always ensued within a few days of the onset of lactation artificially induced during pregnancy by prolactin injections, were presumably not due to the injections of prolactin *per se*.

(*b*) *Effect of prolactin injections on the condition of the mammary glands.* A milk-like fluid was present in the mammary glands of all the female animals—rabbits and monkeys—2–10 days after the beginning of daily prolactin injections. In most cases the amount of fluid which could be squeezed from the mammary glands increased for 2–3 weeks, and in some rabbits a copious secretion of milk was produced. The animals were not milked unless, as in a number of rabbits, it became necessary because of mammary distension. No animal was ever milked on more than two occasions. The mammary glands of all the monkeys ceased to contain fluid after 3–4 weeks' daily injections, but the majority of the rabbit mammae still contained a milk-like fluid, as shown by squeezing and by *post mortem* examination, 18 weeks after the daily injections of prolactin had begun. All the female rabbits used in these experiments were almost certainly parous, but the monkeys were certainly not.

In a number of instances the mammary glands of the male rabbits contained traces of an opalescent fluid after 1–2 weeks' daily prolactin injection. The nipples of both male and female animals appeared to increase substantially in size as the result of the prolactin injections, although, in most instances, the prolactin preparations used possessed no detectable gonadotropic activity.

(*c*) *Antithyrotropic activity of the antiserum to prolactin.* As the prolactin used to prepare the antisera possessed no detectable thyrotropic activity, the antisera might be expected to possess little or no antithyrotropic activity. The rabbit antiserum did, however, possess the power to prevent hypertrophy of the thyroid gland of the young guinea-pig receiving injections of a pituitary thyrotropic preparation containing no detectable amount of prolactin. Thus, when a group of 10 immature guinea-pigs received daily injections for 6 days of 1 mg. of a thyrotropic preparation together with 1 ml. of normal rabbit serum, the average increase in weight of the thyroid glands [method of Rowlands & Parkes, 1934] was $29 \pm 3{\cdot}3$ mg. When, instead of normal rabbit serum, serum from donor rabbits which had received daily prolactin injections for $16\frac{1}{2}$ weeks was given, the average increase in thyroid weight was $8 \pm 2{\cdot}4$ mg. Thus, the serum raised against a prolactin preparation possessing no detectable thyrotropic activity possessed substantial antithyrotropic activity.

(*d*) *Effectiveness of antiserum to ox-prolactin against prolactin from other species.* Although extensive investigations were not made on this point, it may be stated that the antiserum (rabbit) to ox-prolactin inhibited the hypertrophic action on the pigeon crop gland of prolactin from sheep pituitaries.

Discussion

The interpretation of the results of the experiments in which sera were injected daily into lactating mice is a matter of some difficulty, owing to the deaths in the litters of the experimental groups. It seems improbable that these deaths were due to a toxic action of the antisera on the mothers, as very few deaths occurred in the litters whose mothers received daily injections of normal rabbit or normal monkey serum. Another possible explanation is that the mice receiving the antisera provided insufficient milk for the whole litter, and the weakest members of the litters died in the struggle for existence.

Parkes [1926] has carefully studied the question of the growth of young mice according to the size of the litter, considering only those litters in which no deaths occurred. He found that, from birth to 21 days, the average increase in weight of the mice in the litters containing 7 young was only about 75 % of that of the members of litters containing 4. At the end of the present experiments the control litters averaged 6·7 young each, while the litters of the mice receiving rabbit antiserum averaged only 3·6. From the consideration of the size of litter, therefore, the expected increase in the average weight of the young in the litters of mice receiving rabbit antiserum is greater than that actually found in the control group (Fig. 5), and the observed growth of the litters whose mothers received rabbit antiserum is probably significantly subnormal. The sharp rise in the growth curve for this group, beginning on the 19th day, is probably associated with weaning, and provides support for the belief that the amount of milk supplied by the mothers was inadequate for the proper growth of the young. Nevertheless, it is clear that the inhibition of milk secretion brought about by the daily injection of a relatively large amount of the antiserum fell far short of that caused by hypophysectomy [cf. Nelson, 1936]. Many investigators have failed to initiate or reinstate lactation in hypophysectomized animals by the administration of prolactin and even crude pituitary

extracts and implants of whole gland have been ineffective in some instances [cf. Selye *et al.* 1934]. These observations suggest that pituitary substances besides prolactin influence the secretion of milk by the mammary gland. The suggestion has been made that the factor or factors from the anterior lobe concerned with carbohydrate metabolism may play a part in the secretion of milk [Young, 1936; Gomez & Turner, 1936]. The antiserum produced in the present investigation which is active in preventing the pigeon crop-gland response to prolactin, may be lacking in ability to inhibit the action of other factors concerned in milk secretion. In this case the ability of the sera to prevent the pigeon crop-gland response would not necessarily be a measure of its power to inhibit the secretion of milk.

Although the donor rabbits were resistant to the glycotropic action of injected prolactin, no satisfactory evidence was found that this resistance could be transmitted to other animals in the serum of the donor animals. No evidence was obtained that an "antihormone" was present in the serum of the resistant animals, and the cause of their resistance should possibly be sought elsewhere. It cannot be concluded, however, as the result of these experiments, that an antiserum to the glycotropic substance cannot be produced. Investigations with other fractions and with glycotropic preparations from the pituitary glands of species other than the ox may throw light on this question.

The presence of substantial antithyrotropic activity, in the antiserum prepared against a prolactin preparation which contained no detectable thyrotropic activity, may be explicable on the basis of the presence of traces of thyrotropic hormone contaminating the prolactin. In the light of other experiments, however, this explanation appears improbable, and the significance of these observations will be discussed elsewhere. At present it is only desired to put them incidentally on record.

Summary

1. Large doses of ox prolactin possessing no detectable thyrotropic activity were administered daily to rabbits and monkeys for long periods. The serum of animals so injected for 12 weeks or more possessed marked ability to prevent the hypertrophic response of the pigeon crop gland to prolactin.

2. The average weight of the young reared by lactating mice which received daily injections of rabbit antiserum was slightly, but probably significantly, less than that of the young of control animals receiving similar injections of normal serum. This was possibly due to partial inhibition of milk secretion in those animals receiving antiserum.

3. Rabbits which had received daily injections of prolactin for 18 weeks exhibited a normal hypoglycaemic response to insulin after the administration of a potent pituitary glycotropic preparation, i.e. they were insensitive to the glycotropic action of the extract. No evidence was found, however, that this resistance to the glycotropic action of pituitary preparations was transmissible to normal animals by the serum of the resistant rabbits.

4. Although the prolactin used to prepare the sera contained no detectable thyrotropic activity, the antiserum obtained from the rabbits possessed substantial antithyrotropic activity. By thyrotropic activity is meant the ability to induce hypertrophy of the thyroid gland of the immature guinea-pig.

I wish to express my thanks to Dr I. W. Rowlands and Mr M. Chance, who carried out many of the pigeon tests, and to Dr A. S. Parkes for his interest and advice.

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LXXXVIII. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC PRINCIPLE IN HOG'S STOMACH

III. THE INTERACTION OF HAEMOPOIETIN AND PEPSIN WITH MYOGLOBULIN AND CASEINOGEN

By TUDOR STANLEY GEORGE JONES,
WILLIAM SCOTT MINTO GRIEVE and
JOHN FREDERICK WILKINSON

From the Department of Clinical Investigation and Research, the University and Royal Infirmary of Manchester

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Klein & Wilkinson [1933; 1934] described the isolation from hog's stomach of fractions which, when administered orally in relatively small doses, were effective in producing remissions in cases of pernicious anaemia. Further, when these fractions were incubated with beef, a relatively thermostable and therapeutically effective concentrate could be made from the products of digestion. Castle *et al.* [1929; 1930] had suggested that normal gastric juice contained an enzyme, the absence of which from the stomach was an important aetiological factor in pernicious anaemia. Klein & Wilkinson [1934] ascribed an enzymic nature to the substance (haemopoietin) obtained by them from stomach which they considered to be identical with Castle's hypothetical gastric juice enzyme.

The suggestion that the haemopoietic activity of normal gastric juice is due to the presence of an enzyme raises the question of its optimum *p*H; a range of *p*H from 2·5 to 10 has been employed in digestion experiments involving gastric juice and its possible substrates. Lately Castle has exclusively used reactions around neutrality and has claimed [Castle *et al.* 1937] that no activity of mixtures incubated at $p\mathrm{H} < 2{\cdot}5$ has been demonstrated. This is in accordance with our own views and those of Meulengracht [1935] who believes that the enzyme is secreted by the Brunner's glands of the pylorus and duodenum. It thus appears probable that the site of action of haemopoietin is in the neutral or more alkaline regions of the gastro-intestinal tract rather than in the more acid regions of the stomach.

The nature of the substrate has been widely investigated. Castle [1929] first employed beef muscle on account of its accessibility and the fact that Cohn *et al.* [1928] had stated their belief that the liver principle, supposed to be the end product of the enzymic reaction, was a nitrogenous base or a polypeptide. Castle *et al.* [1930] obtained a satisfactory response to the incubation products of normal gastric juice and a crude preparation of the globulin from muscle similar to that described by Edsall [1930]. Using reprecipitated beef muscle globulin, they could produce no remission and thought that this was due to denaturation of the protein. Several other substances have been shown to contain the substrate necessary for the action of the enzyme and it is interesting that liver extract (that is the proteose fraction) has been said by Helmer *et al.* [1933], Reimann [1931] and others to contain this "extrinsic factor".

Griffiths [1934], by incubating beef myoglobulin with normal gastric juice from patients with pernicious anaemia and achlorhydric secondary anaemia at *p*H 6, claimed to have obtained proteolysis with the normal juice only and suggested that the proteolysis was due to interaction between the intrinsic and extrinsic factors. This has been criticized by Emerson & Helmer [1936] who showed that any change in non-protein nitrogen at *p*H 6 could have arisen from peptic hydrolysis. Lasch [1937] claimed to have obtained similar results, using a preparation of washed, dried beef as substrate, but this has not been confirmed by us. More recently still, Taylor *et al.* [1937], reported a progressive change in acid-soluble N when caseinogen was incubated with normal gastric juice at *p*H 7·4, under conditions in which the possible action of pepsin was excluded.

Castle *et al.* [1930] showed that the products of peptic digestion of beef failed to produce remissions in pernicious anaemia. While the peptic and haemopoietic activities of ordinary desiccated whole hog's stomach run parallel [Wilkinson, 1932], active fractions of hog's stomach have been prepared which have little peptic activity when tested by standard methods [Klein & Wilkinson, 1933]. Further, the oral administration of pepsin even in large quantities (up to 30 g. daily) with and without an acid medium has been repeatedly tried by one of us (J. F. W.) in several hundred patients with pernicious anaemia without ever producing any evidence of remission; intramuscular injection of crystalline pepsinogen was also shown to be inactive by Flood & West [1936]. Moreover, the removal of pepsin and rennin from gastric juice by means of various reagents such as caseinogen [Castle *et al.* 1931], calcium triphosphate and Lloyd's reagent [Helmer & Fouts, 1937] or by incubation at *p*H 10 [Flood & West, 1936] failed to remove the intrinsic factor. It is, therefore, quite certain that the intrinsic factor, haemopoietin, is not identical with pepsin.

The experiments described in this paper were designed further to elucidate the enzymic properties of fractions of hog's stomach. To this end we have examined chemically the interaction of these fractions with myoglobulin from beef and with caseinogen and compared the results with those of similar experiments using pepsin. Although pepsin is excluded from consideration as the intrinsic factor (haemopoietin), the properties of the crude fractions containing the latter were found to be so similar to those of pepsin as to merit careful comparison. Preliminary experiments showed that no significant change in amino-N takes place during incubation of myoglobulin with these fractions at various reactions (cf. Taylor *et al.* [1937]; Helmer & Emerson [1937]). We therefore decided to use the N soluble in trichloroacetic acid as a measure of the proteolysis, especially since the liver principle, the postulated end product of the reaction, is a nitrogenous substance soluble in that acid. A discrepancy between our results and the reported value of the Michaelis constant for pepsin [Haldane, 1930] led us further to study the influence of substrate concentration on the kinetics of action of pepsin on caseinogen.

Experimental

The enzyme preparations used in this work were:

(1) *Pepsin* (*Parke Davis 1:10,000*)—contained lactose and was assayed at 1:15,000 by the U.S.P. method. We are indebted to Dr K. Bullock for the assay and gift of this specimen.

(2) *Fraction P5* (Klein & Wilkinson)—this was a fresh preparation made from hog's stomach by the method of Klein & Wilkinson [1933].

(3) *Fraction P5* (*ii*) [Klein & Wilkinson, 1933]. Several different preparations of P5 (ii) were made for the work; careful attention to detail, the precipitation

of P5 (i) at *p*H 3·8 measured by the hydrogen electrode and immediate dehydration of the alcohol precipitate at *p*H 6 with successively stronger solutions of alcohol gave a preparation of twice the enzymic activity of that described by Klein and Wilkinson. The yield of this material was increased by the means quoted to 30 g. from 100 g. of P5. It was found that substantially the same product was obtained if only 2 vol. of 92·5 % (by vol.) alcohol were added to the solution at *p*H 6 after the isoelectric precipitation of P5 (i) and filtration.

(4) *Fraction P5 (i)* (Klein & Wilkinson). In order to obtain a sample of P5 (i) uncontaminated by P5, the solution of P5 in *N*/15 acid was filtered before the addition of alkali to *p*H 3·8. The enzymic activity of this preparation was rather smaller than that of Klein & Wilkinson.

The substrates

(1) *Myoglobulin.* Edsall [1930] has described the preparation of the globulin of beef muscle in solution in a form as nearly as possible unchanged from that in which it occurs in the living tissue. For our work it was originally decided to follow this method as closely as possible (method A), especially as Castle *et al.* [1930] ascribed certain unfavourable results to denaturation of the protein. Later, however, a modified method was adopted (method B), by means of which a considerably larger yield of globulin was obtained. While the globulin fraction obtained by this method would not entirely correspond in physico-chemical properties with that obtained in method A, for our purposes it could be considered the same protein. It has, moreover, the merit of close similarity to the "beef muscle globulin" of Castle *et al.* [1930].

Method A. The cheek and jaw muscle of the ox, obtained within 30 min. of slaughter, is freed from fat and minced, ice being added to keep the tissue cold. The weighed and minced tissue (800–1000 g.) is stirred for 1 hr. with ice-cold water to remove blood, etc., fat being skimmed from the surface of the water. The tissue is removed on a sieve and squeezed to remove excess water and immediately placed in roughly four times its weight of a salt solution of ionic strength 1·3–1·4 and *p*H 7·8 containing 1·2 *M* KCl, 0·05 *M* K_2HPO_4 and 0·01 *M* KH_2PO_4 of 0–5°. After stirring mechanically for 2 hr., the thick suspension is put through a wire sieve to remove connective tissue, allowing adequate time for drainage, the solution being retained tenaciously by the gelatinous tissue. The liquid is filtered on a Büchner funnel through a pad consisting of one Whatman No. 41 filter paper, a 2 cm. layer of paper pulp and a layer of calais sand of approximately the same thickness. The clear filtrate (about 4 l. including some washings) is diluted to 48 l. with water and after 1 hr. the finely suspended protein is removed by a Sharples super-centrifuge. To facilitate separation of the protein from the bowl of the centrifuge, the latter is lined with a sheet of celluloid, which can be removed easily at the end of the operation and from which the protein can be washed by means of buffer solution. The yield of protein is small, amounting to 2–3 g.

Method B. The use of cheek and jaw muscle was recommended by Edsall on the grounds that the protein could be extracted in greater yield and in a less altered condition than if ordinary muscular tissue be used. Assuming the protein we extracted to be myoglobulin, the contrary has been our experience. In place of tissue taken from the freshly killed animal, we have used the cheapest frozen beef with markedly improved yield of protein.

Approximately 1 kg. of minced frozen steak is washed with water at *p*H 5 for 1 hr. and extracted as in method A at *p*H 7–8 for 1 hr. After this extraction

the *p*H is slowly raised to 9 and the mixture stirred for a further hour. The gelatinous tissue is now removed and the fluid portion filtered as before. The filtrate is diluted with water to 20 vol. and brought to *p*H 5. Careful adjustment to *p*H 6 results in a copious precipitate of protein, which flocculates and settles quickly. This is washed by decantation and much of the suspending fluid can be removed by simple filtration by gravity, taking care, however, that the precipitate does not become dry. The protein is soluble in alkaline and acetate buffers (the latter on warming to 40°). From 700 g. of meat there were obtained in one experiment 2·4 l. of 0·7 % protein, i.e. 16·8 g.

A series of suspensions of the finely precipitated protein of strength 0·56 mg. N per ml. (the whole of the N being present as protein) were made in *M*/12 Walpole's acetate buffers *p*H 1·5–6 and in *M*/15 phosphate buffers up to *p*H 9.

(2) *Caseinogen.* This was made from skimmed grade A milk by isoelectric precipitation according to Van Slyke and Baker [1918]. The caseinogen was collected by sedimentation, washed with distilled water twice daily for 4 days, redissolved in *N*/100 NaOH and reprecipitated. The second precipitate was washed as before and filtered off on cheese cloth, the excess fluid being removed on a cheese press. The resultant cake was shaken into a large volume of alcohol and left for several days, the alcohol being removed on a cheese press and replaced by ether and finally benzene and the protein was then dried in a vacuum desiccator. The caseinogen was easily soluble in dilute alkali and by rapidly taking to *p*H 2 with vigorous mechanical stirring and warming to 40°, solutions at that *p*H could be obtained.

For the investigation of the influence of *p*H on the activity of the enzyme preparations, a series of solutions in *M*/12 acetate buffers was made, containing 0·38 mg. N per ml., by diluting a solution prepared as above with *M*/5 acetate buffer and water.

The *p*H of these standard solutions of caseinogen and also of those of myoglobulin was determined by both the quinhydrone and hydrogen electrodes, with excellent agreement.

The p*H activity curves of pepsin and hog's stomach fractions. The determination of activity*

Numerous preliminary experiments were carried out to find suitable conditions for the comparison of activities at different *p*H and the following method was finally adopted.

100 ml. of buffered protein solution (or suspension) were mixed with 10 ml. of the enzyme solution of suitable strength in a 500 ml. bolt-head flask; 15 ml. were removed immediately and run into 10 ml. of 20 % trichloroacetic acid and well mixed. The flasks containing the enzyme-substrate mixtures were placed in a thermostat maintained at $37{\cdot}5 \pm 0{\cdot}05°$. The contents of the flasks were stirred mechanically. Samples (15 ml.) were removed from the flasks at suitable intervals, depending on the concentration of protein and enzyme, and the proteins precipitated as in the initial sample. Next day, the protein precipitate was filtered off on a small Whatman No. 42 filter paper, the first runnings being returned to the filter. No cloudy filtrates were obtained [cf. Taylor *et al.* 1937]. The non-protein N in the filtrate was determined by the micro-Kjeldahl method. Duplicate determinations carried through from the beginning usually agreed to one drop of *N*/100 alkali. The following blanks were carried out at the same time: (1) incubation of the protein with the addition of water in place of the enzyme solution, (2) incubation of the protein with a solution of the enzyme which had been heated at 100° for 10 min., (3) incubation of a solution of enzyme

similar to (2) with water and (4) incubation of a solution of the enzyme with water. It was found that no significant changes in the non-protein N occurred in any of these solutions during 7 hr.

Calculation of activity. The increase in non-protein N over the initial value was found for each sample and plotted against the time at which the sample was taken. Limiting the amount of hydrolysis to a small fraction of the protein present, by a suitable choice of conditions, the velocity curves approximate to straight lines (see Fig. 6). Haldane [1930] and Bodansky [1937] have stressed the importance of using the time taken to produce a given change rather than the change produced in a given time, as a measure of the activity of the enzyme. Where the velocity curves are not straight lines, we have therefore measured the time taken to produce the hydrolysis of one-third of the protein present in the system. The reciprocal of this time gives, in arbitrary units, the activity of the sample of enzyme used. Under conditions where the reaction velocity curves did not approach straight lines, Figs. 1 A and B show the relationship of the activity

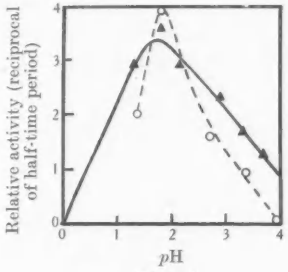

Fig. 1 A.

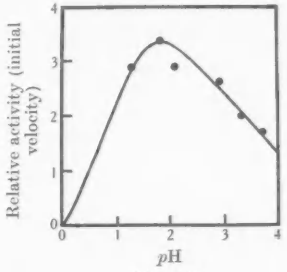

Fig. 1 B.

Fig. 1. Comparison of activity-*p*H curves for the action of pepsin on caseinogen. A, activity calculated from reciprocal of half-time period. B, Calculated from initial velocity. Broken line: curve after Northrop [1922–23].

of pepsin acting on caseinogen to the *p*H of the medium. Fig. 1 A was obtained by plotting the reciprocal of the time taken to hydrolyse half of the protein present against the *p*H, while Fig. 1 B represents the variation of the initial velocity with *p*H. The curves are plotted on arbitrary scales chosen so that the activities at optimum *p*H are approximately the same in the two curves. It is seen that the curves are very similar, the optimum *p*H being 1·6–1·7 and the relative activity is slightly more in the alkaline regions, when calculated by the initial velocity method. For comparison, the broken line in Fig. 1 A shows the results obtained by Northrop [1922–23] for the same system, using a method whereby the unchanged caseinogen was removed from solution, dried and weighed.

Having established the conditions under which straight line reaction velocity curves are produced, we used the initial velocity method. In the absence of this condition, we always obtained the full time course of the reaction and compared the times for a given change.

Reproducibility of p*H-activity curves*

To test the reproducibility of the curves obtained by these methods and to compare the activities of two samples of P5 (ii) prepared under similar conditions at widely separated intervals, we have plotted the curve shown in Fig. 2. The activities of a solution of P5 (ii) of concentration of 0·01 % acting on solutions

of caseinogen containing 0·35 mg. N per ml. at the *p*H shown in the graph were determined simultaneously for each sample. The points for the two samples were obtained at an interval of 14 days. The points relating to sample I (triangles) and sample II (circles) are distributed at random about the smooth curve joining the mean at each *p*H. It is concluded that the method is adequate as far as reproducibility is concerned.

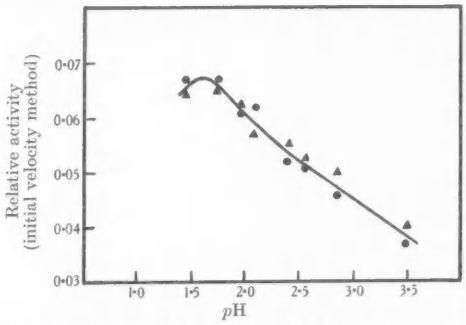

Fig. 2. Activity-*p*H curve of two samples of P5 (ii) acting on caseinogen. Ordinate mg. N produced in 30 min.

Caseinogen as the substrate

The curves in Fig. 3 represent the *p*H-activity relationships of pepsin and various fractions of hog's stomach using caseinogen 0·35 mg. N per ml. as substrate. The curves relate to:

I. Pepsin 0·027 mg. per ml. (triangles)
II. P5 0·10 mg. per ml. (circles)
III. P5 (i) 0·73 mg. per ml. (diamonds)
IV. P5 (ii) 0·10 mg. per ml. (squares)

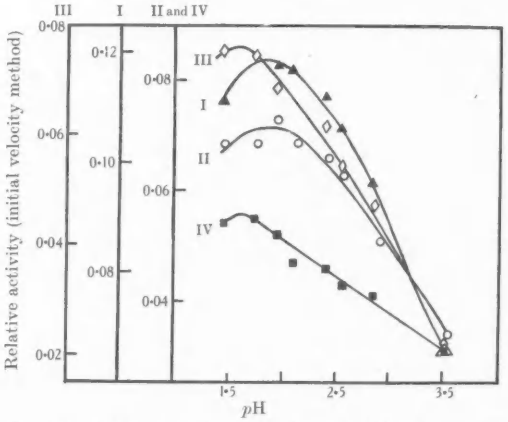

Fig. 3. Comparison of activities of pepsin and hog's stomach fractions acting on caseinogen. Ordinates mg. N produced in 30 min. For explanation see text.

The ordinates are the relative activities calculated as mg. non-protein N per ml. produced in the solution in 30 min. (straight line velocity curves). The scale of the ordinates is equal for all the curves, but the zeros have been chosen so that the position on the graph at *p*H 3·5 is the same for all the preparations studied. The results will be discussed below.

Myoglobulin as substrate

Fig. 4 shows the results using myoglobulin as substrate. The substrate concentration was 0·56 mg. N per ml. and the curves relate to:

I. Pepsin 0·045 mg. per ml. (triangles)
II. P5 0·18 mg. per ml. (circles)
III. P5 (ii) 0·18 mg. per ml. (squares)

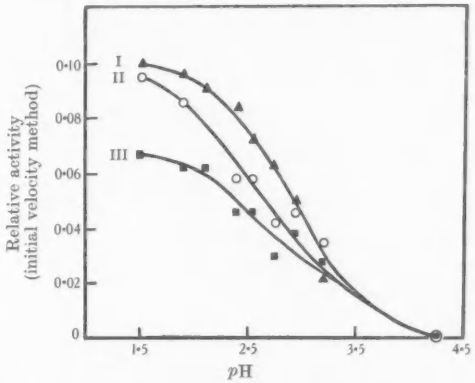

Fig. 4. Comparison of activities of pepsin and hog's stomach fractions acting on myoglobulin. Ordinate mg. N produced in 30 min.

The striking feature of the curves in both figures is their great similarity. While the optimum *p*H is approximately the same for all the preparations studied, there are minor differences between the ratios of the activity at the optimum *p*H to that at another *p*H. By the arrangement of the zeros to make the activity at *p*H 3·5 the same for all the preparations (Fig. 3), the heights of the optimum represent this ratio. This difference in the ratio is less marked with myoglobulin as substrate. In both cases, the curve for P5 occupies a position intermediate between that of pepsin and P5 (ii). This is in accordance with the preparation of P5 (ii) which aims at the removal from P5 of the pepsin which it is known to contain. While the curves shown end at *p*H 3·5 in the case of caseinogen and at 4·3 for myoglobulin, determinations were actually carried out up to *p*H 8·7. Prolonged digestion at the higher *p*H liberated amounts of non-protein N, which were negligibly small compared with the amounts liberated at more acid reactions and which were practically zero on the scale shown in Figs. 3 and 4. In the case of myoglobulin, it is seen from Fig. 4 that the activity is already zero at *p*H 4·3. The results are similar to those of Northrop [1922–23] for the action of pepsin on caseinogen (dotted line in Fig. 1 A), the activity of which becomes zero at *p*H 4 and remains so at higher hydrogen ion concentrations. Under our conditions, in no case was any activity observed at *p*H 7 comparable with that at *p*H 1·5.

The influence of substrate concentration

Substrate. It was found impossible to prepare solutions of myoglobulin of sufficient concentration for this work. The experiments were therefore confined to caseinogen as substrate. For this purpose, caseinogen was precipitated from alkaline solution as a fine suspension, allowed to settle and finally centrifuged at high speed. The caseinogen was then suspended in acetate buffer solutions of the optimum *p*H (1·55) in a series of descending concentrations from 6·2 mg. N per ml.

The velocity curves. Determinations of the velocity curves were carried out for several concentrations of the substrate using 0·18 mg. per ml. of P5 (ii) and 0·03 mg. per ml. of pepsin, exactly as described for the experiments on the *p*H-activity relationships. In the case of the stronger suspensions, since pipetting involved gross errors, we resorted to weighing the substrate suspension. Duplicate determinations of reaction velocity showed excellent agreement of an order

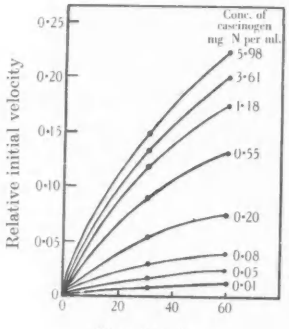

Fig. 5.

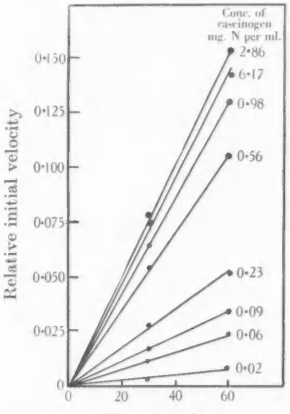

Fig. 6.

Fig. 5. Effect of concentration of caseinogen on initial velocity of the action of pepsin. Ordinate mg. N produced in 30 min.

Fig. 6. Effect of concentration of caseinogen on initial velocity of the action of P5 (ii). Ordinate mg. N produced in 30 min.

comparable with that of the more dilute suspensions. After precipitation of the protein with trichloroacetic acid, the mixtures were diluted with an appropriate solution of trichloroacetic acid in such a proportion that the amount of precipitate per unit volume was approximately the same for all solutions. This was intended to minimize errors due to unequal distribution of non-protein N between the solution and the protein precipitate. The non-protein N in the filtrate from the diluted deproteinized sample was then determined as usual.

The velocity curves for the two enzyme preparations studied are shown in Figs. 5 and 6 and the initial velocities corresponding to these curves are shown as points in Figs. 7 and 8, plotted against the logarithm of the concentration of substrate (calculated as protein N per ml.).

The action of pepsin on caseinogen

The unbroken curve shown in Fig. 7 is plotted from the equation of Michaelis & Menten [1913], Km having the value 0·316 mg. N per ml. which corresponds to a concentration of 0·20 % assuming caseinogen contains 15·62 % N. It is evident that in the range of concentration studied, there is excellent agreement between the experimental results and the theory of Michaelis. Haldane [1930] quotes a value of 4·5 % for Km in the action of pepsin on ovalbumin, derived from certain results of Northrop [1919–20]. Allowing for the differences between suspensions of caseinogen and ovalbumin, it is unlikely that the value of Km for the latter should be more than 20 times our value for the former. Inspection of Northrop's paper fails to reveal sufficient data on which the calculation of Km could be based.

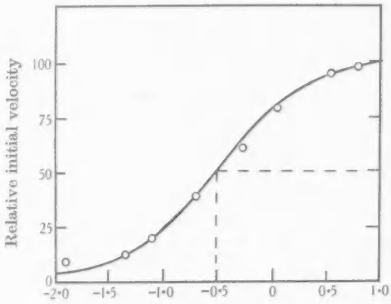

Fig. 7.

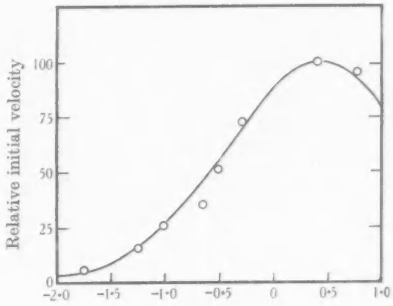

Fig. 8.

Fig. 7. Relationship of initial velocity and logarithm of substrate concentration (mg. caseinogen—N per ml.) for the action of pepsin.

Fig. 8. Relationship of initial velocity and logarithm of substrate concentration (mg. caseinogen—N per ml.) for the action of P5 (ii).

The action of P5 (ii) on caseinogen

The theory of Michaelis and Menten covers the data obtained for this system up to a concentration of about 1 mg. N per ml.; above this concentration the velocity is less than would be expected on the simple theory. Haldane [1930] has attempted to explain the inhibiting effect of high concentrations of substrate by combination of the compound ES with further substrate to give ES_2, ES being the only compound which breaks down to form the end products of the reaction. The proportion of such unions to form ES_2 increases with substrate concentration and since they are unfruitful from the point of view of proteolysis, the velocity falls off. Consideration of the kinetics of this system leads to the equation

$$\frac{v}{ke} = \frac{1}{1 + \frac{K_1}{x} + \frac{x}{K_2}},$$

where the additional constants K_1, K_2, k and e are respectively the Michaelis constants of the reactions $E+S \rightleftharpoons ES$, $ES+S \rightleftharpoons ES_2$, the velocity constant of the slow reaction $ES \rightleftharpoons E+P$ (where P = products) and the total concentration of the enzyme in the system.

The unbroken curve in Fig. 8 has been fitted to the points by suitable choice of k' according to the equation

$$\frac{v}{k'}=\frac{1}{1+\frac{0{\cdot}398}{x}+\frac{x}{17{\cdot}78}}.$$

The behaviour of the enzyme accords well with the theory of Haldane and the values of K_1 and K_2 are 0·398 and 17·78 mg. N per ml. respectively. The value of the constant for the reaction $E+S \rightleftharpoons ES$, viz. $K_1=0{\cdot}398$ mg. N per ml. or 0·256 % caseinogen concentration, is 28 % higher than that for the action of pepsin on caseinogen.

Discussion

In the above experiments, haemopoietin has been assumed to be a proteolytic enzyme and has been compared with pepsin but our attention lately has been increasingly drawn to the possibility that haemopoietin acts, not on protein, but on some other nitrogenous substance, possibly a product of other (peptic?) proteolytic action. If the assumption be true that it is a proteolytic enzyme, three possibilities present themselves: (*a*) that haemopoietin is identical with pepsin, (*b*) that it differs from pepsin but has some very similar properties, and (*c*) that the fractions used in the work contain so little of the active enzyme that its properties are masked by those of pepsin.

The first is easily disposed of since the non-identity of the two enzymes has been abundantly proved by us and others in a large series of clinical tests.

The expected optimum *p*H for the action of haemopoietin depends on our views as to its site of action; if the stomach is the usual site, then the optimum would reasonably be expected to be similar to that of pepsin; on the other hand the possibility that haemopoietin may be secreted in the pylorus and even in the Brunner's glands of the duodenum suggests that the enzyme might act at neutral or even slightly alkaline reactions. It must be admitted that our own experience, like that of Castle suggests an optimum action around the neutral point. Consequently, the question arises whether, in view of the absence of any proteolysis at *p*H > 4 comparable with that at *p*H 1·5, we are actually measuring the action of the haemopoietin known to be present in the preparations. The close similarity of the *p*H-activity curves, in spite of minor differences, and of the kinetics of reaction of preparations of haemopoietin and pepsin, supports the view that we are measuring the action of pepsin alone. While a careful attempt has been made in the preparation of P5 (ii) to remove all pepsin, it is possible that the salt produced by neutralization of the HCl used for solution of the P5 might aid in keeping some of the pepsin in solution [cf. Fenger & Andrew, 1927]. Contamination of the haemopoietin of P5 (ii) with pepsin is therefore to be considered when explaining the similarity of the properties of the two enzymes under discussion.

The possibility that haemopoietin may not be a proteolytic enzyme, but may act on protein split-products has not been investigated with any thoroughness. Castle *et al.* [1937] have brought forward clinical evidence that the *in vivo* action of gastric juice on the products of peptic digestion of beef produces a remission in cases of pernicious anaemia. It is to be presumed that here the haemopoietin acts on some peptone-like substance, although we must not lose sight of the possibility that sufficient residual protein may remain as a substrate for the action of the haemopoietin.

SUMMARY

1. The effect of *p*H on the production of non-protein N in the interaction of beef muscle globulin and caseinogen with pepsin and preparations of hog's stomach has been analysed.

2. The effect of the substrate concentration on the hydrolysis of caseinogen by these preparations has been determined and shown to be in accord with the theory of Michaelis and Menten for the action of pepsin, and with Haldane's modification of the theory for an inhibited reaction in the case of the stomach fraction P5 (ii). The Michaelis constant for the former reaction is 0·316 mg. N per ml. or 0·20 % caseinogen while for the latter, the constants K_1 and K_2 have the values 0·398 mg. N per ml. and 17·78 mg. N per ml. respectively.

3. The bearing of these results on the nature of the enzyme haemopoietin is briefly discussed.

We are indebted to the Medical Research Council for grants, under which this work has been carried out, and to Messrs Boots Pure Drug Co. Ltd., Nottingham, for generous supplies of fraction P5.

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LXXXIX. FAT METABOLISM IN FISHES

XII. SEASONAL CHANGES IN THE COMPOSITION OF HERRING FAT

By JOHN ARNOLD LOVERN

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research

(*Received 17 February 1938*)

It is well known that the fat content of herring varies over a wide range and extreme values may be as low as 1 % or as high as 30 %. Lovern & Wood [1937] made a study of the seasonal nature of these variations and showed that following minimal values about April, there was a rapid rise in fat content during late May and June to maximal values in July. After this point had been reached the length of time that a high fat content was maintained depended on when the fish spawned; relatively high values were maintained until spawning was imminent, when cessation of feeding caused a rapid drop in the fat content. The rapid rise in June was associated with the intensive feeding which then takes place, food organisms being very abundant at that time. In winter, scarcity of food, and possibly lack of appetite, result in a steadily falling fat content to the minimum values in April.

In the present paper it is shown that the fat of herring varies in composition as well as in quantity, and the variations of composition are likewise of a seasonal nature.

Experimental

Herring were obtained at such seasons as seemed likely to be of particular interest from the aspect of fat metabolism. Thus a sample was taken in April, when the fat content should be minimal. Three samples were taken during the period of rapid rise in fat content (June and July) and two samples were obtained in October, one from spent fish and the other from fish only $\frac{1}{2}$–$\frac{3}{4}$ ripe [cf. Lovern & Wood, 1937]. Of these latter samples the spent fish contained considerably less fat than the others. All the foregoing fish were adults; in addition, immature herring caught in April were examined.

Actually the above samples were selected from a considerable number of batches of herring which were tested for fat content, the choice being guided by the results of this test. Each sample was biologically uniform in itself but it would have been difficult to secure racial uniformity from sample to sample and no attempt was made to do so. Unfortunately, a really lean April sample was not forthcoming, but it seems very doubtful whether such a sample would have yielded any further information.

The fish were gutted and the whole of the remaining tissues, including bones and head, were minced and well mixed and a sample was extracted to give sufficient fat for analysis. Each batch consisted of about 20 herring, selected on a basis of biological uniformity as already mentioned. The particulars of these samples are given in Table I. The fats were examined by the ester fractionation method and the fatty acid compositions are given in Table II. Degrees of average unsaturation are expressed as lack of hydrogen, as in former papers.

Table I. *Particulars of samples of herring*

Sample no.	Month caught	% fat	Iodine value of fat
1	April	8·2	115·5
2	June	10·7	144·2
3	June	15·7	154·3
4	July	20·7	152·5
5	October	18·8	138·6
6	October	12·0	129·9
7*	April	4·6	147·9

* Immature fish.

Table II. *Composition of fatty acids of herring fats (wt. %)*

	Saturated			Unsaturated				
Sample	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
1	8·0	15·7	0·2	—	4·6 (−2·6 H)	22·2 (−2·9 H)	22·0 (−3·9 H)	27·3 (−4·2 H)
2	7·3	16·7	Trace	0·6	7·5 (−2·7 H)	21·1 (−3·3 H)	27·3 (−4·8 H)	19·5 (−5·7 H)
3	7·5	12·8	0·1	0·3	7·0 (−3·0 H)	21·1 (−4·8 H)	30·0 (−5·2 H)	21·2 (−4·8 H)
4	8·3	12·1	0·3	0·5	6·4 (−3·4 H)	21·0 (−4·5 H)	28·3 (−5·5 H)	23·1 (−4·6 H)
5	7·3	13·0	Trace	0·8	4·9 (−2·7 H)	20·7 (−4·2 H)	30·1 (−4·6 H)	23·2 (−4·3 H)
6	6·6	13·7	0·5	0·2	4·9 (−2·8 H)	16·3 (−3·6 H)	28·7 (−4·4 H)	29·1 (−4·1 H)
7	5·8	12·4	0·6	—	4·7 (−3·0 H)	17·8 (−3·9 H)	31·1 (−4·3 H)	27·6 (−4·8 H)

Discussion

Before considering the variations that are evident from sample to sample in Table II, there are several features to be noted which are peculiar to all the herring fats. These features may be regarded as specific peculiarities of the herring. In a previous paper [Lovern, 1937] the average composition of a marine fish fat was arrived at by means of frequency curves. Such a composition would involve the following approximate amounts of unsaturated acids: C_{16} 10 %, C_{18} 25 %, C_{20} 25 %, C_{22} 15 %. These figures correspond to the maxima on the frequency curves and, of course, a fair variation from them must be allowed before a fat could be described as abnormal. The fats in Table II are quite within the normal range for C_{18} and C_{20} acids but they undoubtedly have an abnormally high content of C_{22} acids and this feature may probably be considered as a characteristic property of herring fat. In this connexion, however, it may be noted that far more liver fats than body fats from marine fish were incorporated in the curves referred to above. There may be a tendency towards higher proportions of C_{22} acids in the body fats from marine fish, such as, for instance, turbot and halibut [Lovern, 1937]. The content of C_{16} unsaturated acids is on the low side but the frequency curve for the proportion of these acids in a marine fish fat is unsymmetrical. The true maximum probably corresponds to between 5 and 10 % of the acids. If total C_{16} acids (both saturated and unsaturated) are considered, the peculiarity of the herring fats is not marked, the average for a marine fish fat being 20–25 %. In the writer's opinion it is doubtful how far a low content of C_{16} unsaturated acids should be regarded as a specific characteristic of herring fat.

There is a further peculiarity in all the fats in Table II. Many of the degrees of average unsaturation are abnormal in all the samples, irrespective of variations from sample to sample. Average values for the unsaturation of the different acid groups in a marine fish fat were quoted in the paper already referred to [Lovern, 1937] and may be given again: C_{16}, $-2{\cdot}0$ to $-2{\cdot}5$ H; C_{18}, $-2{\cdot}6$ to $-3{\cdot}3$ H; C_{20}, $-5{\cdot}5$ to $-6{\cdot}5$ H; C_{22}, $-7{\cdot}0$ to $-9{\cdot}5$ H. It will be seen that all the herring fats have C_{16} acids of abnormally high degrees of unsaturation and C_{22} acids of abnormally low degrees of unsaturation. Most of them have unusually unsaturated C_{18} acids and unusually saturated C_{20} acids. Taking them as a whole it would be fair to say that the specific features of herring fat include abnormality of unsaturation for all the acid groups, unusually high values for C_{16} and C_{18} acids and low values for C_{20} and C_{22} acids.

Apart from these general features there are variations from one sample to another. The variations in the proportions of the different acids are in certain cases significant; C_{20} acids range from 22·0 to 31·1 % and C_{22} acids from 19·5 to 29·1 %. The cause of the variations is not clear. The fact that large amounts of C_{22} acids are found in samples 1, 6 and 7, which are from fish which had undergone partial starvation, is in harmony with previous evidence [Lovern, 1934, 2]. In starving salmon it was shown that fatty acids of low molecular weight were more readily mobilized than those of higher molecular weight, leading to a progressive accumulation of the latter. However, there is no indication of a preferential mobilization of the smaller molecules in this case, e.g. C_{14} acids. The largest amounts of C_{16} unsaturated acids are found in the feeding fish (nos. 2, 3 and 4) and the smallest amounts in the starved fish, but this is not the case with the total C_{16} acids, and, as has been shown previously, it is the total C_{16} acids which must be considered in view of the interconvertibility of saturated and unsaturated derivatives.

Whatever the cause of these variations in the proportions of the various acids, the variations themselves, although real enough, are not of a large order, and do not strikingly differentiate the samples. On the other hand the samples cover a wide range as regards the average degrees of unsaturation of the acids. C_{16} acids range from $-2{\cdot}6$ to $-3{\cdot}4$ H, C_{18} from $-2{\cdot}9$ to $-4{\cdot}8$ H, C_{20} from $-3{\cdot}9$ to $-5{\cdot}5$ H and C_{22} from $-4{\cdot}1$ to $-5{\cdot}7$ H. These variations are not erratic. They are lowest in starved fish and highest in feeding fish. Sample 7 will be considered separately; in the others the unsaturation of C_{16} acids rises steadily as the fish feed and reaches a maximum when the fat content is at a maximum, after which it falls away. The other groups of fatty acids follow a similar course except that in C_{18} acids and especially in C_{22} acids the maximum unsaturation is reached earlier than the maximum fat content.

During the period of intensive feeding by herring in June, fat is being ingested at a very high level. A major ingredient of the diet is the copepod *Calanus finmarchicus*, which has a high fat content. Moreover, *Calanus* fat is highly unsaturated, especially as regards C_{18} acids ($-5{\cdot}1$ H) [Lovern, 1935]. The C_{20} and especially C_{22} acids of herring fat always have such low degrees of unsaturation, even when the herring are rapidly ingesting copepod fat containing highly unsaturated C_{20} and C_{22} acids, that it seems certain that hydrogenation of these acids must have taken place [cf. Lovern, 1937]. If hydrogenation of C_{18} acids also takes place, it does so to a less extent than in C_{20} and C_{22} acids.

In the writer's opinion the observed variations are brought about as follows. The herring prefers a depot fat with comparatively saturated C_{20} and C_{22} acids and to this end ingested C_{20} and C_{22} acids are partially hydrogenated. In June

the fish ingest highly unsaturated fat at an abruptly increased and very high level. It seems only natural that the hydrogenation mechanism should prove temporarily unequal to such a sudden change in diet. The unsaturation of all acid groups would tend to rise. Then the hydrogenation mechanism would function more actively and it seems likely that it would operate preferentially on the C_{22} acids. It is in respect of the C_{22} acids that the specific peculiarity of the herring is most marked when compared with the average marine fish. Thus, long before the period of intensive feeding is over, the rate of saturation of C_{22} acids is sufficient to cope with the ingested fat (the drop from sample 2 to 3, during the period of intensive feeding, is striking). The saturation of the other acids is not so urgent, although steady hydrogenation of C_{20} acids must be going on, as, in spite of the fact that most of the fat in sample 4 must be fat deposited during the intensive feeding period, its unsaturation is only $-5{\cdot}5$ H as opposed to $-7{\cdot}8$ H in *Calanus* fat [Lovern, 1935]. In this connexion it should be noted that sample 1 with 8·2 % of fat is not representative of the minimum fat content which herring attain before the commencement of intensive feeding. A figure of 2–3 % is much nearer the average. Thus it is clear that the fat of sample 4 is mainly fat which has been recently deposited.

It has been mentioned that *Calanus* fat has particularly highly unsaturated C_{18} acids ($-5{\cdot}1$ H). It seems from what has already been said that the herring does not require a relatively saturated C_{18} acid mixture—in fact from the high state of unsaturation of C_{18} acids in sample 5, long after the period of intensive feeding has passed, it appears that an unusually high degree of unsaturation of these acids can be tolerated. However, there is presumably a limit above which the unsaturation cannot rise without an attempt being made to modify it. In sample 3, when rapid ingestion of fat has gone on some time and is still going on, the C_{18} acids of the herring fat have almost reached the state of unsaturation of *Calanus* fat. Evidently there has been no attempt so far to hydrogenate them. After this level has been attained, however, there is a drop (sample 4), whilst still more fat is being ingested. The limit appears to have been reached in sample 3 and hydrogenation of the C_{18} acids has then commenced.

The C_{16} unsaturated acids are unique in that they have a higher level of unsaturation than *Calanus* fat ($-2{\cdot}4$ H). Their increase in unsaturation during the feeding period cannot, therefore, be due to ingestion of food fat at a higher level than the herring metabolism can deal with. In a previous paper [Lovern, 1937] it has been suggested that hydrogenation and dehydrogenation of fats in fish are brought about by a reversible enzyme system. The writer's opinion is that dehydrogenation of C_{16} acids goes on during intensive feeding at the same time as hydrogenation of the other acids. The C_{16} acids may indeed provide part of the hydrogen required for hydrogenation of the other acids.

The compositions of samples 5 and 6 are suggestive. Both of these are from fish caught at the same period, long after intensive feeding has ceased. The herring of sample 6, however, having recently spawned, had undergone partial starvation through loss of appetite, whilst the fish of sample 5 had gone on feeding at the normal summer and autumn level. Sample 5 had not lost much fat but sample 6 had been considerably depleted. Both sets of fish had had the same period in which hydrogenation could go on, yet in sample 6 the acids (apart from C_{16}) are more saturated than in sample 5. This suggests a preferential utilization of the more unsaturated acids. There is a long gap in time between samples 4 and 5. In view of the rapidity with which hydrogenation must have been taking place to cope with the high rate of fat ingestion, it seems likely that when intensive feeding ceased in July the herring fat would soon

reach the required degree of saturation. Probably the degree of saturation found in sample 5 was attained long before October and then remained more or less stationary. The fall in sample 6 compared with sample 5 can only be associated with the lower fat content and not with the length of time that hydrogenation has gone on—hence the conclusion that selective utilization of the more unsaturated constituents has taken place. The C_{16} acids are interesting in this respect. The extra unsaturation acquired during the intensive feeding period seems to be lost very rapidly and these acids have reached the minimum unsaturation required by the herring in sample 5. Any further drop would be prevented either by dehydrogenation or by a cessation of selective utilization. If there is a tendency (as found in the salmon) for fatty acids of smaller molecular size to be most readily utilized as fuel, and if in addition the herring preferentially utilizes the more unsaturated constituents of the fat, then the very sudden drop in unsaturation of the C_{16} acids is readily understandable.

The degree of unsaturation is still lower in sample 1 than in sample 6, except in the case of the C_{16} and C_{22} acids which show little change. The lower limit of unsaturation for both these acids seems to have been almost reached in sample 5.

It should be noted that in the case of starving salmon already referred to there was no evidence of a preferential utilization of the more unsaturated acids.

The immature herring of sample 7 should be compared with those of sample 1, caught at the same period. It will be seen that the degrees of unsaturation are higher throughout. This condition of higher unsaturation in young specimens may be true for many species. For instance, young salmon have much more highly unsaturated fat than adult salmon [Lovern, 1934, 3], and the fat of the foetus of a porpoise was found to be much more highly unsaturated than the maternal fat [Lovern, 1934, 1].

Summary

The fats from samples of herring caught at different seasons have been analysed and the fatty acid compositions recorded.

An abnormally high content of C_{22} acids, unusually unsaturated C_{16} and C_{18} acids and unusually saturated C_{20} and C_{22} acids are considered as characteristic features of the fats of the species.

Unsaturation of the different acid groups varies progressively from sample to sample. It is suggested that rapid ingestion of highly unsaturated fat during June causes a temporary unbalancing of fat hydrogenation in the herring. A temporary rise in unsaturation results, but hydrogenation is soon modified to meet the situation. C_{16} acids are dehydrogenated instead of being hydrogenated whilst C_{18} acids are not markedly affected over a large range.

It is suggested that in fasting herring the more highly unsaturated constituents of the fat are preferentially utilized.

Immature herring had a more unsaturated fat than adult herring caught at the same period.

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XC. THE FAT OF THE GREEN TURTLE

By THOMAS GEORGE GREEN and THOMAS PERCY HILDITCH
From the Department of Industrial Chemistry, University of Liverpool

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The green turtle (*Chelone mydas* Linn.) is fairly widely distributed in many parts of the tropics, especially perhaps in the Indian and Pacific Oceans. Hornell [1927] states that young green turtles feed on fine confervae and on the leaves of sea-grass (*Cymodocea*); the adult animals live almost entirely upon this sea-grass, but occasionally also on Sargasso weed. They are however occasionally carnivorous, and sometimes eat shellfish, notably immature oysters. The flesh of the turtle contains a certain amount of fat, which is marketed to some extent. The present communication describes the component acids present in a specimen of the fat of green turtle from the Seychelles Islands.

The only other detailed study of the fat of which we are aware was made by Tsujimoto [1937], who examined the oil from green turtles taken in the Ogasawara Islands, Japan. Tsujimoto found that oleic acid was the chief constituent, whilst myristic acid was present in fairly large amounts, together with palmitic and stearic acids, small proportions of highly unsaturated acids (C_{20} and probably C_{18}), and possibly lauric and hexadecenoic acids. Our results confirm the qualitative statements of Tsujimoto in most respects, but differ somewhat in that we are able to show that lauric, myristic and palmitic acids are each present in very similar proportions, these three saturated acids together amounting to half of the total fatty acids.

The general analytical characteristics of the fat now examined are compared in Table I with those recorded by Tsujimoto [1937] and the Imperial Institute

Table I. *Analytical characteristics of turtle fats*

(i) *Green turtle*, Chelone mydas

Source	Seychelles Is.	Japan	Ceylon	Panama
Observer	Present work	Tsujimoto [1937]	Imp. Inst. [1937]	Imp. Inst. [1937]
Sap. equiv.	265·9	272·0	269·7	262·3
Acid value	3·2	1·4	1·1	0·6
I.V.	66·1	64·1	68·2	61·5
Unsaponifiable	1·5 %	0·5 %	0·5 %	0·5 %

(ii) *Other species of turtle*

Species	*Dermochelys coriacea*	*Dermochelys schlegelii*		*Lepidochelys olivacea*	Unspecified		
Source	Ceylon	Japan		Ceylon	West Africa	Ceylon	Egypt
Observer	Imp. Inst. [1937]	Imp. Inst. [1937] Carapace	Tsujimoto [1920] Flesh	Imp. Inst. [1937]	Lee [1935]	Imp. Inst. [1937]	Imp. Inst. [1937]
Sap. equiv.	280·5	283·5	308·8	292·5	268·0	267·3	262·9
Acid value	17·1	1·6	2·0	0·4	2·0	1·5	—
I.V.	103·8	98·9	128·1	148·7	64·6	65·5	57·2
Unsaponifiable	1·8 %	3·3 %	—	1·3 %	0·6 %	0·7 %	—

[1937] for other specimens from *Chelone mydas*, and with the data of Tsujimoto [1920], Lee [1935] and the Imperial Institute [1937] for fats from other species of sea-turtle.

It is evident from Table I that the fat of the green turtle is characterized by comparatively low equivalents and I.V., the respective figures lying within the limits of 263–270 and 62–68.

Component fatty acids of green turtle fat

We submitted the acids from about 300 g. of the fat to analysis by the ester-fractionation method, after resolving them by lead salt-alcohol separation into 26·7 % of "solid" acids (methyl esters, sap. equiv. 265·5, I.V. 2·8) and 73·3 % of "liquid" acids (methyl esters, sap. equiv. 273·0, I.V. 86·7). The methyl esters of the "solid" acids were distilled from a Willstätter bulb, whilst those of the "liquid" acids were fractionated through an electrically heated and packed column; the ester-fractions obtained are shown in Table II.

Table II

Methyl esters of "solid" acids

(Willstätter bulb)

Primary fractionation				Refractionation of S 1			
No.	g.	Sap. equiv.	I.V.	No.	g.	Sap. equiv.	I.V.
				S 11	3·59	227·4	—
				S 12	3·25	233·4	—
				S 13	3·22	240·2	—
				S 14	3·13	245·0	—
S 1	33·81	250·0	0·5	S 15	3·16	247·6	—
				S 16	3·09	253·0	—
				S 17	3·25	259·0	1·0
				S 18	4·31	267·3	2·1
				S 19	2·86	274·7	6·3
S 2	6·76	267·0	1·3				
S 3	6·22	271·0	1·4				
S 4	7·34	272·5	1·9				
S 5	6·15	276·0	2·5				
S 6	9·98	283·8	5·2				
S 7	6·49	303·2	19·4				

Methyl esters of "liquid" acids

(Electrically heated and packed column)

No.	g.	B.P./0·1 mm. °C.	Sap. equiv.	I.V.
L 1	2·98	83–88	212·4	3·5
L 2	12·45	88–90	214·2	1·5
L 3	4·37	90–96	216·5	3·3
L 4	2·08	96–108	229·4	10·8
L 5	3·48	108–110	240·6	14·5
L 6	5·02	110–126	249·8	33·6
L 7	6·08	126–128	269·2	76·1
L 8	4·01	128–130	269·8	74·0
L 9	6·05	130–142	278·9	78·2
L 10	13·71	142–144	290·7	91·5
L 11	14·44	144	294·8	94·9
L 12	13·10	144	294·5	96·2
L 13	9·26	144–145	295·2	95·7
L 14	7·99	145–147	295·9	100·0
L 15	4·24	147–155	301·9	145·3
L 16	8·63	Residue	361·9	244·2*

* Esters, freed from unsaponifiable, sap. equiv. 328·6, I.V. 254·7.

The acids from ester-fraction L 2, after a single crystallization from 70 % alcohol, melted at 43·5° (unchanged on admixture with an authentic specimen of lauric acid).

Acids from fractions (e.g. L 12, L 13) obviously consisting almost entirely of unsaturated C_{18} esters were submitted to bromination and oxidation. On addition of bromine in ethereal solution to 4·61 g. of these acids, there were obtained 0·07 g. of bromo-additive products insoluble in boiling ether (softening without melting at 250°), 0·04 g. insoluble in cold ether (M.P. 232°), 0·14 g. soluble in ether but insoluble in petrol (M.P. 250° indef.), and 7·37 g. of oily products soluble in both ether and petrol. Of polyethenoid C_{18} acids, therefore, a small amount of highly unsaturated C_{18} acids was detected, but no ordinary linoleic acid; this agrees with the observations of Tsujimoto [1937], to the effect that a small proportion of the highly unsaturated C_{18} acids is present, and that by far the greater part of this group consists of oleic acid. This conclusion is further confirmed by the results of alkaline permanganate oxidation of another specimen of these acids (4·4 g.), when 0·07 g. of polyhydroxystearic acids, which melted indefinitely at 140–150°, was obtained, together with 2·96 g. of slightly impure 9:10-dihydroxystearic acid (M.P. 127–128°). The absence of linoleic acid and the presence of small amounts of polyethenoid C_{18} acids places the unsaturated C_{18} acids of the turtle oil definitely in the "aquatic fat" category.

Addition of bromine to the acids (2·07 g.) from the residual fraction L 16 yielded 1·37 g. of bromo-additive products insoluble in ether, with M.P. above 250°, and characteristic of polyethenoid C_{20} or C_{22} acids.

The data in Table II lead to the composition of the total fatty acids of the turtle fat given in Table III.

Table III. *Component acids of green turtle fat*

	"Solid" (26·7 %)	"Liquid" (73·3 %)	Total	% (wt.)	% (mol.)
Saturated:					
As decanoic	—	0·15	0·15	0·2	0·3
Lauric	1·11	12·14	13·25	13·3	16·9
Myristic	6·53	4·03	10·56	10·6	11·9
Palmitic	13·86	3·09	16·95	17·0	17·0
Stearic	4·09	—	4·09	4·1	3·7
Unsaturated:					
As tetradecenoic	—	1·32	1·32	1·3	1·5
Hexadecenoic	—	7·72	7·72	7·8	7·8
C_{18} unsaturated	0·54	38·83	39·37	39·6	35·8 (− 2·2 H)
C_{20-22} unsaturated	0·57	5·50	6·07	6·1	5·1 (− 6·3 H)
Unsaponifiable	—	0·52	0·52	—	—

The composition of this fat presents several points of interest. The major component acid is oleic, and the presence and proportions of palmitic, hexadecenoic and unsaturated C_{20-22} acids are consistent with those of fats of other amphibians recently studied by various workers [Klenk *et al.* 1933, 1935; Hilditch & Paul, 1937]; the proportions of the unsaturated C_{16} and C_{20-22} acids are intermediate between those of depot fats of land and of marine animals. The unusual feature in this fat is the occurrence, in addition to palmitic acid, of significant quantities of its next lower natural homologues, myristic and (especially) lauric acid; traces of a saturated acid of lower mol. wt. than lauric acid are also present. The combined molar percentage of saturated acids is almost exactly 50 % of the total fatty acids.

Whilst it is not by any means unique to find lauric acid as a constituent of marine animal fats—it is present, for example, in sperm head oil [Hilditch & Lovern, 1928] and porpoise depot fats [Lovern, 1934]—it is usually accompanied by subsidiary amounts of dodecenoic acid, which in this case is not present. On the other hand, it has not been observed in the other amphibian or reptile fats hitherto investigated, and its presence in quantities equivalent to that of palmitic acid is definitely remarkable. The diet of the green turtle, which has already been mentioned, affords little, if any, reason to suppose that this acid has been derived therefrom by assimilation.

Glyceride structure of the green turtle fat

The somewhat peculiar composition of the mixed acids suggested that a general examination of the component glycerides present in the fat might prove interesting. A quantity of the green turtle fat (609 g.) was separated by successive crystallizations from acetone at 0° into a small portion of solid fat and a large portion completely liquid at room temperature:

	g.	I.V.	% (wt.)	% (mol.)
Less soluble portion (A)	53	16·1	8·7	9·3
More soluble portion (B)	556	70·8	91·3	90·7

Less soluble glycerides (*A*). The amount available was insufficient to permit of component fatty acid determinations being made on the whole and on the fully saturated portion of A. The fully saturated components were therefore isolated and analysed, the component acids in the non-fully saturated portion being later determined by difference (from the analyses of the total fatty acids of the turtle fat and those of the acids in fraction B and the fully saturated part of fraction A). The less soluble portion A (47·0 g.), oxidized in acetone solution with powdered $KMnO_4$, gave 27·6 g. of fully saturated material (I.V. negligible, acid value 1·3, sap. equiv. 251·8), corresponding to a fully saturated glyceride content in this portion of the fat of 57·7 % (wt.) or 58·1 % (mol.), and equivalent to 5·4 % (mol.) of fully saturated glycerides in the whole turtle fat. Ester-fractionation of the component acids of the fully saturated portion showed a molar content of 24·7 % lauric, 27·5 % myristic, 35·7 % palmitic and 11·3 % stearic acids, together with traces of decanoic and oleic acids. These figures (as will be seen from Table IV, below) show that the remainder of the portion A of the fat consisted wholly of mono-unsaturated (C_{16}, C_{18}, C_{20}) disaturated (C_{12}, C_{14}, C_{16}, C_{18}) glycerides.

More soluble glycerides (*B*). 150 g. of this portion were hydrolysed, and the component fatty acids determined by lead salt separation and ester-fractionation exactly as in the case of the whole fat (Tables II and III). The final results of the analysis are given in Table IV.

Another portion (52·1 g.) was oxidized twice in acetone solution with powdered $KMnO_4$, when 2·6 g. of fully saturated material (I.V. 0·4, acid value 14·6, sap. equiv. 239·5) were obtained. This corresponds to 4·1 % (wt.) or 4·6 % (mol.) of the glycerides in B, and to 4·2 % (mol.) of the whole fat. It will be noticed that the average equivalent (239·4) of these fully saturated glycerides is lower than that (251·8) of those in the less soluble, crystalline fraction. This indicates that, as would be expected, the mixed glycerides of lauric, myristic, palmitic and stearic acids which separated from acetone solution contain more combined palmitic and stearic acids than the more soluble fully saturated glycerides left in solution, in which lauric and myristic acids evidently predominate.

Finally, a portion of B (50 g.) was completely hydrogenated and some of the product (40·6 g.) systematically crystallized from anhydrous ether; the least soluble fraction obtained (6·9 g.) had an equivalent of 293·1, whilst that of the next fraction in order of solubility (5·3 g.) was 275·6. These figures indicate the presence of about 10 % (mol.) of tri-C_{18} glycerides in B, or about 9 % (mol.) of tri-C_{18} glycerides in the whole fat.

Table IV summarizes the information obtained from this part of the investigation.

Table IV. *General glyceride structure of green turtle fat (% mol.)*

	Less soluble portion A (9·3 %)		Most soluble portion B†
Component acids	Fully saturated (5·4 %)	Saturated-* unsaturated (3·9 %)	(90·7 %)
Saturated:			
As decanoic	Trace	Trace	Trace
Lauric	1·3	0·6	15·3
Myristic	1·5	0·6	9·8
Palmitic	1·9	1·3	13·8
Stearic	0·7	0·2	2·8
Unsaturated:			
Tetradecenoic	—	—	1·5
Hexadecenoic	—	0·5	7·3
C_{18}	—	0·3	35·5 (− 2·2 H)
C_{20-22}	—	0·4	4·7 (− 6·6 H)

* Component acids calculated by difference; this portion of the fat is wholly composed of mixed mono-unsaturated-disaturated glycerides.

† Portion B contained fully saturated glycerides (in which lauric and myristic acids predominated) equivalent to 4·2 % (mol.) of the whole fat; it also contained about 9 % (mol.) of tri-C_{18} glycerides.

The total fully saturated glycerides in the green turtle fat amount to 9·6 % (mol.), and the data suggest that the individual saturated acids are present in this group in much the same proportions as those in which they occur in the whole fat. The somewhat similar quantity of tri-C_{18} glycerides present is probably not wholly in the form of triolein, but may also include a certain proportion of stearodiolein. Although, in the nature of the case, it has not been possible to obtain much detailed information as to the component glycerides of the more soluble portion B of the turtle fat, the data revealed in Table IV suffice to show that the fat is a typical mixture of mixed glycerides in which for the most part the fatty acids are distributed as widely or evenly as possible throughout the triglyceride molecules. It appears quite clear, at all events, that the lauric acid, which is an unusual acid to encounter in this class of fat, is distributed indiscriminately with the more commonly occurring acids throughout the mixed glycerides.

Summary

The component acids and glycerides of the fat of the green turtle (*Chelone mydas* Linn.) have been examined. In most respects the component acids are qualitatively and quantitatively similar to those which have been observed in depot fats of other amphibious animals; but they are unusual in that they include about 17 % (mol.) of lauric acid and more myristic acid (12 % mol.) than is customary. The total saturated acids, indeed, amount to 50 % of the whole of the fatty acids; a similar high content of saturated acids (usually confined to palmitic and stearic) has, however, been recorded previously in the

case of the fats from a few species of tropical fish [Tsujimoto, 1932; Wang & Kan, 1936]. Oleic acid is the dominant unsaturated acid, but unsaturated C_{16}, C_{20} and C_{22} acids are also present in proportions similar to those previously observed in other amphibian depot fats.

The glycerides are a complex mixture in which for the most part the acidic components (including the lauric acid) are distributed fairly evenly and widely in combination with the glycerol molecules. The fat includes nearly 10 % of fully saturated glycerides (chiefly mixed lauro-myristo-palmitins) and about the same quantity of tri-C_{18} glycerides, some of which is probably stearodiolein. Most of the glycerides must include at least one oleic group associated with one or (more frequently) two acyl groups originating from the rest of the mixed fatty acids.

We are indebted to Mr F. M. Dyke of Messrs Lever Brothers, Ltd., who very kindly procured for us the specimen of green turtle oil from the Seychelles; and to Mr W. H. Pedelty for assistance with one of the ester-fractionation analyses.

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XCI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LVII. FUMIGATIN (3-HYDROXY-4-METHOXY-2:5-TOLUQUINONE) AND SPINULOSIN (3:6-DIHYDROXY-4-METHOXY-2:5-TOLUQUINONE), METABOLIC PRODUCTS RESPECTIVELY OF *ASPERGILLUS FUMIGATUS* FRESENIUS AND *PENICILLIUM SPINULOSUM* THOM

By WINSTON KENNAY ANSLOW and HAROLD RAISTRICK

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

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During the routine examination of a number of cultures of fungi isolated by Mr L. D. Galloway [1936] from Indian soil, our colleague, Mr G. Smith noticed that the metabolism solution of one of them—an indubitable strain of *Aspergillus fumigatus* Fresenius—changed from yellowish brown to a strong purple colour on making alkaline.

We have investigated the reason for this unusual colour change and have shown that it is due to the presence of a hitherto undescribed mould metabolic product which we have called *fumigatin*.

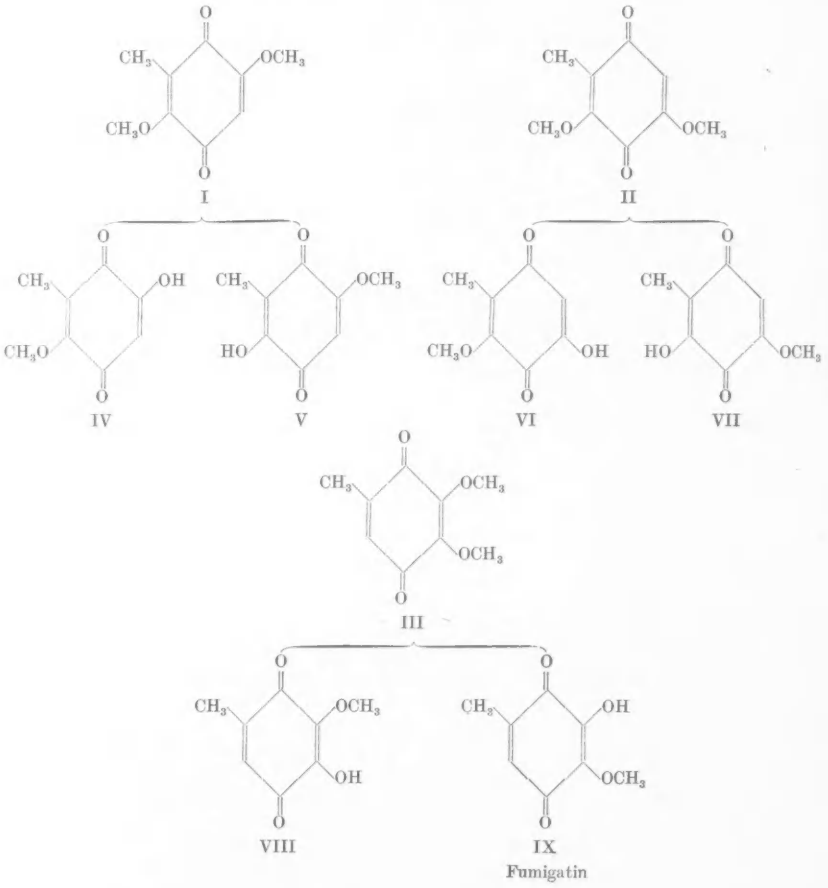
Fumigatin, $C_8H_8O_4$, was isolated as maroon-coloured needles, M.P. 116°. It contains one methoxy group (Zeisel), one methyl group (Kuhn-Roth) and one hydroxy group since it forms a monoacetate and a monomethyl ether. It is without question a monohydroxymonomethoxytoluquinone and the synthesis of fumigatin methyl ether proved conclusively that fumigatin itself is a *p*-quinone and not an *o*-quinone.

There are three possible dimethoxy-2:5-toluquinones, I, II and III, each of which has recently been synthesized here [Anslow *et al.* 1938]. I may arise by methylation of either of the monohydroxymonomethoxy-2:5-toluquinones IV or V, II from VI or VII and III from VIII or IX.

Anslow *et al.* [1938] showed that I melts at 104–105°, II at 125° and III at 59°. Since fumigatin methyl ether melts at 59°, and since its M.P. is not depressed on mixing with III, it follows that fumigatin methyl ether must be III and hence that fumigatin itself must be either VIII or IX.

The ethyl ether of VIII was synthesized by the following series of reactions. Vanillin was reduced to creosol (4-hydroxy-3-methoxytoluene) which was nitrated to 5-nitrocreosol by the method of Oberlin [1925]. This was ethylated to give *5-nitro-3-methoxy-4-ethoxytoluene* which on reduction yielded *5-amino-3-methoxy-4-ethoxytoluene*. On oxidizing this substance *3-methoxy-4-ethoxy-2:5-toluquinone*, i.e. the ethyl ether of VIII was obtained. It melted at 50° and on reduction with sodium hydrosulphite gave the corresponding crystalline quinol *3-methoxy-4-ethoxytoluquinol*, M.P. 64°. On the other hand fumigatin gave an

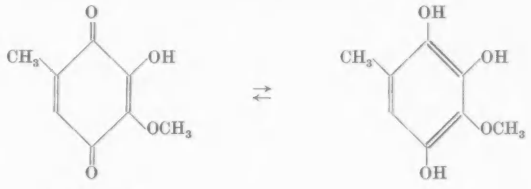
ethyl ether which, on reduction with sodium hydrosulphite, gave the corresponding crystalline quinol, M.P. 55–56° alone, depressed to 46–47° in admixture with 3-methoxy-4-ethoxytoluquinol. Hence fumigatin cannot be VIII and must



be IX, i.e. *3-hydroxy-4-methoxy-2:5-toluquinone*. The quinol, M.P. 55–56°, derived by reduction of the ethyl ether of fumigatin is therefore *4-methoxy-3-ethoxytoluquinol*.

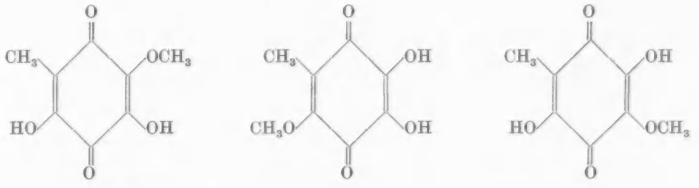
The probable significance of fumigatin in the metabolic processes of the particular strain of *A. fumigatus* used in this investigation is indicated by the fact that we have also isolated from the same freshly separated metabolism solution not only fumigatin, but also its reduction product, the quinol *3-hydroxy-4-methoxytoluquinol* (X). Since these two substances have been shown to be

readily interconvertible, and since they are both undoubted metabolic products, it is difficult to resist the conclusion that they function as an oxidation-reduction system in the life processes of the mould.



Fumigatin X

Birkinshaw & Raistrick [1931] reported the isolation from cultures of strains in the *Penicillium spinulosum* Thom series of a new mould metabolic product which was unnamed at that time, but for which the name *spinulosin* is now proposed. Spinulosin was shown to be a dihydroxymonomethoxytoluquinone and, assuming it to be a *p*-toluquinone, it followed that it must have one of the three formulae XI, XII or XIII.



XI XII XIII

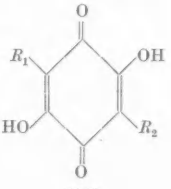
Spinulosin

We have now shown that fumigatin may be readily converted *in vitro* into spinulosin as follows. Fumigatin was submitted to the Thiele-Winter [1900] reaction when a colourless tetraacetate was obtained identical with the tetra-acetate obtained by simultaneous reduction and acetylation of spinulosin. The tetraacetate from fumigatin was hydrolysed to yield the corresponding tetra-hydroxymethoxytoluene, which was oxidized, by passing air through its alkaline solution, to give a good yield of spinulosin.

In the Thiele-Winter reaction, which many substituted benzoquinones undergo, a nuclear —CH group, together with both —CO groups and any —C(OH) groups, are acetylated to —C.O.OC.CH_3. Hence, since it is now known that spinulosin is a *p*-quinone, it follows that spinulosin must be a hydroxyfumigatin, the new hydroxyl group occupying the only available position 6; i.e. spinulosin is *3:6-dihydroxy-4-methoxy-2:5-toluquinone* (XIII). The same conclusion was reached by Aulin & Erdtman [1937] by comparing the colour of an alkaline solution of spinulosin with that of certain synthetic dihydroxy-2:5-toluquinones.

The structure assigned to spinulosin brings it into close relationship with certain other naturally occurring derivatives of 2:5-dihydroxy-1:4-benzoquinone,

the constitutions of which have recently been settled. Thus, taking structure XIV as the general formula for these compounds,



XIV

R_1=methyl and R_2=methoxy in spinulosin. In embelic acid (embelin) the active principle of the berries of *Embelia ribes*, Hasan & Stedman [1931] showed that R_1=H and R_2=*n*-lauryl. Kögl and his co-workers have shown that in polyporic acid, isolated from the agaric *Polyporus nidulans* Fr., $R_1=R_2$=phenyl [1926], and in atromentin, which occurs in the agaric *Paxillus atromentosus* Batch, $R_1=R_2$=*p*-hydroxyphenyl [1928].

Experimental

Culture

The culture used throughout the work was, morphologically, undoubtedly a strain of *Aspergillus fumigatus* Fresenius. It was received in January 1936 from Mr L. D. Galloway, who isolated it from Indian soil [Galloway, 1936]. It bears Mr Galloway's catalogue No. CH 3 and the L.S.H.T.M. Cat. No. A 46. No fumigatin could be detected among the metabolic products of five other strains of *A. fumigatus*, i.e. L.S.H.T.M. Cat. Nos. 47, 88, Ac 15, Ac 70 and Ac 71.

Cultural conditions

The culture medium used throughout was a Raulin-Thom solution of the following composition: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; $FeSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml. This medium was distributed in 350 ml. amounts in batches of 100 1 l. conical flasks, sterilized, sown with a spore suspension of *A. fumigatus* A 46, and incubated at 24°.

After 31–37 days' incubation the metabolism solution, *p*H 3·3 and still containing 0·5–1 % of sugar, was separated by filtration from the mycelium, which was greyish white in colour, with occasional grey-green sporing patches and with a pale pink to purple reverse. The metabolism solution was yellowish brown in colour.

Fumigatin is present in the metabolism solution partly in the oxidized (quinone) form and partly in the reduced (quinol) form. This is evident since both forms were in fact isolated from the same metabolism solution. It is also indicated by the fact that the freshly separated metabolism solution, when strongly acidified and treated with KI, liberates I equivalent to 4–5 ml. *N*/100 I per 100 ml., while after vigorous aeration it liberates I equivalent to 8–10 ml. *N*/100 I per 100 ml. Since fumigatin in the oxidized (quinone) form is stable in air whilst its reduced (quinol) form is very unstable, it was in general isolated in the quinone form.

Isolation and purification of fumigatin

The filtered metabolism solution was vigorously aerated for 1 hr. by which time the iodine titration had reached a constant value. It was acidified with concentrated HCl (30 ml. per 7 l. of medium corresponding to 20 flasks) and each batch of 7 l. was extracted 8 times with 2 l. $CHCl_3$. This laborious procedure was unfortunately necessary since evaporation of the metabolism solution, even *in vacuo* at 40–45°, caused decomposition of the fumigatin. The $CHCl_3$ extracts were evaporated *in vacuo* (bath temperature not >45°) and the concentrated extracts were allowed to evaporate to dryness in air in open dishes, when reddish brown, crystalline, but somewhat sticky, residues were obtained. The average yield of crude fumigatin so obtained was about 5 g. per 100 flasks. The crude material was dried *in vacuo* over conc. H_2SO_4 and was extracted in a Soxhlet apparatus with light petroleum (B.P. 40–50°) when it was obtained as a maroon-coloured crystalline dry powder (yield about 2 g. of almost pure material per 100 flasks). This was further purified by recrystallization from boiling light petroleum (B.P. 40–50°) in which it is only sparingly soluble (0·17 g. in 200 ml. boiling light petroleum). It separated as maroon-coloured needles, M.P. 116°. For analysis a sample was sublimed in a high vacuum. No decomposition occurred and the M.P. was unchanged.

Isolation of reduced fumigatin (dihydrofumigatin) (3-hydroxy-4-methoxytoluquinol) (X)

The metabolism solution from 20 flasks (residual glucose = 0·66 %) was strained through muslin, acidified with conc. HCl (30 ml.) and extracted four times with 2 l. of ether. The ethereal extracts were evaporated and a portion of the concentrated extract was dried *in vacuo* in a sublimation tube and sublimed twice in a high vacuum. All these operations were performed quickly and as far as possible in the absence of air. The sublimate consisted of a mixture of reddish brown crystals (fumigatin) and a colourless semi-solid material. It was dissolved in ether, and washed with freshly prepared aqueous $NaHCO_3$ which removed fumigatin. The ethereal solution was evaporated to dryness and resublimed. The sublimate was washed with a little CCl_4 which removed traces of impurity and resublimed giving almost colourless needles, M.P. 99–101°. (Found: C, 56·93; H, 6·16 %. $C_8H_{10}O_4$ requires C, 56·45; H, 5·93 %.) The M.P. of a mixture of this substance and a freshly made specimen of dihydrofumigatin (M.P. 100–101°) prepared as described on p. 693 was 100–101°. The two substances also gave the same colour reactions.

General properties of fumigatin

Fumigatin crystallizes in maroon-coloured needles, M.P. 116°, and has the empirical formula $C_8H_8O_4$. (Found on two different samples: C, 57·34, 57·29; H, 4·72, 4·65; OCH_3, 18·5, 18·4 %; N, nil. Mol. wt. cryoscopic in dioxane (Dr A. E. Oxford), 161. $C_8H_8O_4$ requires C, 57·12; H, 4·80; 1 OCH_3, 18·5 %. Mol. wt. 168.) All micro-analyses were carried out by Dr G. Weiler, Oxford.

In a Zerewitinoff determination (Roth), fumigatin afforded 1·2 active H atoms in pyridine at 20° and 1·4 at 95°. On oxidation with chromic acid (Kuhn-Roth method) evidence of one side-chain methyl group was obtained. (Found: 98·8 and 99·9 % of 1 $C_2H_4O_2$.)

Fumigatin readily sublimes, without decomposition, in a high vacuum. It is readily soluble in acetone, ether, chloroform, benzene, ethyl acetate and alcohol, fairly soluble in water and slightly soluble in light petroleum.

It gives the following reactions:

(*a*) A solution in absolute alcohol gives with one drop of *N*/2 $FeCl_3$ an intense purple-black colour which changes to light brown on the addition of water, the latter colour being similar to that given on the addition of $FeCl_3$ to an aqueous solution of fumigatin.

(*b*) With *N* NaOH it gives a colour indistinguishable from that of dilute $KMnO_4$. This colour changes to brown on standing overnight.

(*c*) With aqueous $NaHCO_3$, in which it is immediately soluble, it gives a colour indistinguishable from that of dilute $KMnO_4$. This colour is unchanged overnight.

(*d*) With cold conc. H_2SO_4 it immediately gives a brown colour changing to cherry red after 2 min., and becoming an intense permanganate colour after about half an hour.

(*e*) It dissolves readily in aqueous $Na_2S_2O_4$ to give an almost colourless solution from which dihydrofumigatin may be readily extracted by ether (see later).

(*f*) It liberates I from an acidified solution of KI.

(*g*) It does not react with *o*-phenylenediamine in ether solution standing over anhydrous Na_2SO_4, and can be recovered unchanged. Hence it is probably not an *o*-quinone.

Derivatives of fumigatin

(*a*) *Action of diazomethane.* Fumigatin (0·2 g.), dissolved in ether, was treated with ethereal diazomethane. There was immediate and vigorous evolution of nitrogen and the brownish red solution lightened in colour to orange. The yellow crystals (0·15 g.) separating on removal of most of the solvent were recrystallized from ether and were obtained as lemon-yellow needles, M.P. 93°. (Found: C, 53·46, 53·70; H, 5·15, 5·05; N, 12·6, 12·5; OCH_3, 27·5, 28·0 %. $C_{10}H_{12}O_4N_2$ requires C, 53·56; H, 5·40; N, 12·5; $2OCH_3$, 27·7 %.) This substance has obviously arisen from fumigatin by the methylation of one OH group and the addition of the elements of diazomethane CH_2N_2, with the probable formation of a pyrazole complex—a reaction of frequent occurrence with certain types of quinones.

(*b*) *Fumigatin monomethyl ether* (*3:4-dimethoxy-2:5-toluquinone*) (*III*). Fumigatin (0·2 g.) was dissolved in acetone (5·0 ml.) and methyl sulphate (1·0 ml.) was added. The mixture was boiled for 2½ hr., during which time K_2CO_3 (1 g.) was added in four or five lots. The mixture was cooled, diluted with ether and the K_2SO_4 and excess K_2CO_3 removed by filtration. The solvents were evaporated, leaving a dark-coloured oil which was extracted with boiling light petroleum, B.P. 40–50°. The light petroleum was removed and the residue distilled in a high vacuum giving a reddish brown oil which crystallized later. This separated from light petroleum in long red needles, M.P. 59°. The yield was small. The M.P. was not depressed on mixing with a synthetic specimen of 3:4-dimethoxy-2:5-toluquinone, M.P. 59° [Anslow *et al.* 1938]. (Found: C, 59·29; H, 5·58; OCH_3, 33·2 %. $C_9H_{10}O_4$, i.e. the monomethyl ether of $C_8H_8O_4$, requires C, 59·30; H, 5·53; 2 OCH_3, 34·1 %.)

(*c*) *Fumigatin monoacetate* (*3-acetoxy-4-methoxy-2:5-toluquinone*). Fumigatin (0·2 g.) was dissolved in acetic anhydride (2 ml.) by gentle warming. The solution was cooled and 2 drops conc. H_2SO_4 were added. The mixture was heated but not boiled for a few seconds, when the colour changed from blood red to brownish yellow. The crude acetate (0·14 g.) separating on addition of ice and water was recrystallized from light petroleum (B.P. 40–50°) as canary yellow

rosettes of needles, M.P. 95–96°. A further 0·14 g. was obtained from the filtrate by extraction with ether. (Found: C, 57·42, 57·36; H, 4·96, 4·83; OCH_3, 14·2, 14·5 %. $C_{10}H_{10}O_5$, i.e. the monoacetate of $C_8H_8O_4$, requires C, 57·12; H, 4·80; 1 OCH_3, 14·8 %.)

(*d*) *Dihydrofumigatin* (*3-hydroxy-4-methoxytoluquinol*). Fumigatin (0·2 g.) was shaken for a few moments with $Na_2S_2O_4$ (4 g.) in water (20 ml.) when an almost colourless solution was obtained. This was extracted 4 times with an equal volume of ether and the ether removed, leaving a syrupy residue (0·2 g.), which was dried in a high vacuum at 70°, when it crystallized. The bath temperature was then raised to 90–100°, when the quinol sublimed in colourless micro-crystals, M.P. 100–101°. (Found: C, 56·39, 56·28; H, 5·77, 5·73; OCH_3, 18·3, 18·1 %. $C_8H_{10}O_4$ requires C, 56·45; H, 5·93; 1 OCH_3, 18·2 %.)

Dihydrofumigatin is very readily soluble in water giving a colourless solution which quickly becomes pink. The aqueous solution gave the following reactions:

(i) No precipitate and doubtful absorption with bromine water.

(ii) With $NaHCO_3$ a colour indistinguishable from that of $KMnO_4$ solution and from that given by fumigatin itself with $NaHCO_3$.

(iii) With aqueous $FeCl_3$ a yellow changing to a dark brown colour.

When 2*N* NaOH solution is added drop by drop to the quinol in substance or in concentrated solution, a transient bright green colour is formed, quickly becoming olive green with a purple layer on the surface. With excess NaOH an intense purple colour is formed.

A solution of dihydrofumigatin in absolute alcohol gives with *N*/2 $FeCl_3$ first a brownish yellow colour which, on addition of more $FeCl_3$, suddenly becomes an intense purple-black exactly similar to that given by an alcoholic solution of fumigatin.

Attempts to obtain a crystalline acetyl derivative of dihydrofumigatin have been unsuccessful. In different experiments fumigatin was reduced with $Na_2S_2O_4$ and with zinc dust and acetic acid, and the resultant quinol was acetylated in one case with pyridine and acetic anhydride, and in the other with sodium acetate and acetic anhydride. In both cases there was obtained a very viscous colourless oil which resisted all attempts to crystallize it. Further, a quantitative acetylation of pure dihydrofumigatin by the Peterson-West [1927] method (acetic anhydride in pyridine), while proving conclusively the presence of three acetylatable OH groups (found: 43·4 % $CH_3.CO$; $C_{14}H_{16}O_7$, i.e. the triacetate of $C_8H_{10}O_4$, requires 43·6 %) resulted in a triacetate which proved to be a viscous, colourless, uncrystallizable oil.

(*e*) *Fumigatin monoethyl ether* (*4-methoxy-3-ethoxy-2:5-toluquinone*). Fumigatin (0·168 g. 1/1000 g.-mol.) was dissolved in absolute alcohol (2 ml.) and to the brownish red solution were added 10 ml. of *N*/10 ethyl alcoholic NaOH. The colour changed to an intense purple. 10 ml. of *N*/10 ethyl alcoholic $AgNO_3$ were now added and the Ag salt of fumigatin was precipitated as a purple-black solid, which was separated, washed with alcohol and then with ether. While still damp with ether 1 ml. of freshly distilled ethyl iodide was added together with 10 ml. of ether. An immediate reaction occurred at room temperature, and after 1 hr. the mixture was filtered. The ethereal filtrate was shaken with aqueous $NaHCO_3$ to remove a little unetherified fumigatin, dried and evaporated. The orange red viscous residue, consisting of crude fumigatin ethyl ether, was sublimed in a high vacuum and gave an orange-coloured oil which partly crystallized at 0°. However, because of the small amount available, and because of its unsatisfactory diagnostic value for comparison with synthetic material, it was converted into the corresponding quinol.

(*f*) *Dihydrofumigatin ethyl ether* (*4-methoxy-3-ethoxytoluquinol*). An ethereal solution of sublimed fumigatin ethyl ether, reddish brown in colour, was shaken with aqueous $Na_2S_2O_4$ when the ether layer quickly became colourless. It was separated and the aqueous layer was thoroughly extracted with ether. The combined ether extracts were dried, the solvent removed and the residue sublimed in a high vacuum. On resublimation it was obtained as colourless plates, M.P. 55–56°. A mixture with synthetic 3-methoxy-4-ethoxytoluquinol (M.P. 64°, see below) melted at 46–47°. (Found: C, 60·94, 61·08; H, 7·13, 7·08; total alkoxy calculated as OCH_3, 31·5 %. $C_{10}H_{14}O_4$ requires C, 60·57; H, 7·12; 1 OCH_3+1 OC_2H_5, calculated as % OCH_3, 31·3 %.)

Synthesis of 3-methoxy-4-ethoxy-2:5-toluquinone, i.e. the ethyl ether of VIII and of the corresponding quinol, 3-methoxy-4-ethoxytoluquinol

5-Nitro-3-methoxy-4-ethoxytoluene. Vanillin was reduced to creosol which was nitrated to give 5-nitrocreosol by the method of Oberlin [1925]. A mixture of 5-nitrocreosol (20 g.), diethyl sulphate (20 ml.), K_2CO_3 (50 g.) and toluene (200 ml.) was refluxed for 3 hr. A further 20 ml. of diethyl sulphate were added in portions during the next hour and the heating was continued for 7 hr. in all. Toluene was added to the reaction mixture which was thoroughly washed with 2 % NaOH and dried. The toluene was removed and the residue distilled *in vacuo*. The yellow distillate quickly crystallized, B.P. 165–170°/18 mm. Yield, 13·4 g. A small sample, sublimed in a high vacuum, gave an almost colourless sublimate, M.P. 59°. (Found: C, 57·00, 56·97; H, 5·96, 6·12; N, 6·59, 6·57 %. $C_{10}H_{13}O_4N$ requires C, 56·85; H, 6·21; N, 6·64 %.)

5-Amino-3-methoxy-4-ethoxytoluene. Concentrated HCl (39 ml.) and water (13 ml.) were added slowly to a mixture of granulated tin (18·2 g.), alcohol (26 ml.) and 5-nitro-3-methoxy-4-ethoxytoluene (13 g.). When the evolution of hydrogen had moderated, the mixture was refluxed for 1 hr. The alcohol was removed by distillation *in vacuo* and, after cooling, NaOH (32·5 g.) in water (130 ml.) was added. The amine was extracted with ether and remained after removal of the ether as a pale yellow oil (9·1 g.) which was oxidized to the quinone without further purification.

3-Methoxy-4-ethoxy-2:5-toluquinone. The above amine (9·1 g.) was dissolved in a cold mixture of conc. H_2SO_4 (35 ml.) and water (110 ml.), placed in a freezing mixture and treated with $Na_2Cr_2O_7$ (5·5 g.) in water (33 ml.) slowly with constant stirring. The following day a further 11 g. of $Na_2Cr_2O_7$ in 66 ml. of water were added under the same conditions. After 3 hr. at room temperature the mixture was extracted with ether. On removal of the solvent, 2·7 g. of a dark reddish brown oil remained, which crystallized later at 0°. A portion was sublimed in a high vacuum, dissolved in ether, and the ethereal solution extracted with aqueous $NaHCO_3$ to remove a little impurity, which coloured the aqueous layer deep purple. The ether layer was dried, the solvent removed and the residue sublimed in a high vacuum in long orange-red needles, M.P. 50°. (Found: C, 61·50, 61·30; H, 6·27, 6·30; total alkoxy calculated as OCH_3, 32·4, 32·0 %. $C_{10}H_{12}O_4$ requires C, 61·19; H, 6·17; 1 OCH_3, +1 OC_2H_5, calculated as % OCH_3, 31·6 %.)

3-Methoxy-4-ethoxytoluquinol. 0·2 g. of the above sublimed quinone was dissolved in ether and the ether solution shaken with aqueous $Na_2S_2O_4$, when it rapidly became colourless. It was separated and the aqueous layer was thoroughly extracted with ether. The combined ether extracts were dried, and the solvent removed leaving an oil which later crystallized. The crystals were purified by sublimation in a high vacuum to give colourless prisms, M.P. 64°. Yield almost

quantitative. (Found: C, 60·54, 60·44; H, 6·97, 7·05; total alkoxy calculated as OCH_3, 32·2, 31·8 %. $C_{10}H_{14}O_4$ requires C, 60·57; H, 7·12; 1 OCH_3+1 OC_2H_5, calculated as % OCH_3, 31·3 %.)

Thiele-Winter [1900] reaction on fumigatin. Preparation of 2:3:5:6-tetraacetoxy-4-methoxytoluene

Fumigatin (0·5 g.) was dissolved, at room temperature, in 7·5 ml. of a mixture of acetic anhydride (30 ml.) and conc. H_2SO_4 (1 ml.). The brownish red solution which quickly changed to light brown in colour, after 4 days at room temperature, was poured into ice and water. The crude acetyl compound (0·84 g.) was separated, dried and recrystallized from absolute alcohol, with a little charcoal, in colourless crystals separating in a manner reminiscent of ammonium chloride. (Found: C, 54·44, 54·39; H, 5·25, 5·23; OCH_3, 9·5, 9·7 %. $C_{16}H_{18}O_9$ requires C, 54·23; H, 5·12; OCH_3, 8·8 %.)

2:3:5:6-Tetraacetoxy-4-methoxytoluene melts at 192–192·5°. A freshly isolated specimen of the tetraacetate, prepared by simultaneous reduction and acetylation of spinulosin from *P. spinulosum* [Birkinshaw & Raistrick, 1931] also melted at 192–192·5°. A mixture of the two substances melted at the same temperature.

Conversion of fumigatin into spinulosin (i.e. 3:6-dihydroxy-4-methoxy-2:5-toluquinone (XIII)

The tetraacetyl derivative (0·8 g.), prepared from fumigatin as described in the previous section, was hydrolysed by boiling, in an atmosphere of N_2, for ¾ hr., with 10 ml. of a mixture of methyl alcohol (30 ml.) and conc. H_2SO_4 (1 ml.); the solvent was then evaporated *in vacuo* and replaced by water. The aqueous solution was thoroughly extracted with ether, the ethereal solution dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The brownish residue, consisting of crude 2:3:5:6-tetrahydroxy-4-methoxytoluene, was dissolved in water (25 ml.) to give a brownish solution. 2*N* NaOH (2·2 ml.) was added to give a definite alkaline reaction and air was rapidly bubbled through. The solution quickly became intensely purple. When the colour appeared to have reached a maximum, a slight excess of 2*N* HCl was added when spinulosin crystallized as a purple-black microcrystalline powder which was separated and dried (0·19 g.). A further 0·05 g. was isolated by ether extraction of the mother liquors. It was purified for analysis by crystallization from toluene and sublimation in a high vacuum. (Found: C, 52·16, 52·39; H, 4·45, 4·39; OCH_3, 17·6, 17·9 %. $C_8H_8O_5$ requires C, 52·17; H, 4·38; OCH_3, 16·9 %.)

This sublimed sample melted at 201°. A freshly sublimed sample of spinulosin isolated from cultures of *P. spinulosum* also melted at 201°. A mixture of the two substances melted at the same temperature.

Each of these specimens gave a bluish purple colour with *N* NaOH, the colour being bluer in shade than $KMnO_4$ solution, a pure and intense blue colour with conc. H_2SO_4 and an intense rich brown colour in alcoholic solution with $FeCl_3$. The colours given by the natural and synthetic specimens of spinulosin in each of the above colour reactions were indistinguishable from each other.

Summary

A hitherto undescribed mould metabolic product *fumigatin*, $C_8H_8O_4$, and its reduction product, $C_8H_{10}O_4$, have been isolated from cultures of *Aspergillus fumigatus* Fresenius grown on Raulin-Thom medium. Fumigatin has been shown to be *3-hydroxy-4-methoxy-2:5-toluquinone* and its reduction product to be

3-hydroxy-4-methoxytoluquinol. It is suggested that these two substances function as an oxidation-reduction system in the vital processes of this mould.

Fumigatin has been converted, *in vitro*, into *spinulosin*, a metabolic product of *Penicillium spinulosum* Thom, described by Birkinshaw & Raistrick [1931]. Spinulosin has been shown to be *6-hydroxyfumigatin*, i.e. *3:6-dihydroxy-4-methoxy-2:5-toluquinone*.

We desire to thank Dr H. Erdtman, College of Technology, Stockholm, and our colleague, Dr A. E. Oxford, for many valuable suggestions during the course of this work.

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XCII. NOTE UPON THE PREPARATION OF CRUDE COCARBOXYLASE FROM VITAMIN B_1 BY YEAST

By HENRY WULFF KINNERSLEY and RUDOLPH ALBERT PETERS

From the Department of Biochemistry, Oxford

(*Received 15 February 1938*)

Lohmann & Schuster [1937, 1, 2] isolated cocarboxylase from yeast, and proved that this was a pyrophosphoric ester of vitamin B_1. Wishing to obtain crude carboxylase from small amounts of yeast before the details of their complete paper [1937, 2] appeared, we found that baker's yeast aerated in phosphate solution (containing pyrophosphate) with addition of synthetic vitamin B_1 chloride hydrochloride readily gave cocarboxylase. Subsequently pyrophosphate proved to be unnecessary. The cocarboxylase was extracted by heating the yeast and was precipitated from the extract by neutral lead acetate following Auhagen. It seems clear therefore that it is the combined form of this vitamin, first described by Kinnersley & Peters [1928] as constantly precipitable by neutral lead acetate. Since the preliminary account of our work [1937], there have appeared papers by Euler & Vestin [1937] and Tauber [1937] claiming synthesis with dried yeast, and by Stern & Hofer [1937] reporting a very small synthesis with phosphorus oxychloride.

We are describing our latest methods for preparing these crude solutions because they may be of value to others. The pyrophosphoric ester of the vitamin can be detected by the formation of blue fluorescence by treatment with dilute ferricyanide in alkaline solution; this blue fluorescence does not pass into an *iso*- or *n*-butyl alcohol layer. Unlike vitamin B_1 also, as stated by Lohmann and Schuster, it gives no formaldehyde *azo*-reaction.

Experimental

Requirements. Baker's yeast 10 g.; *M*/10 phosphate buffer (KH_2PO_4, Na_2HPO_4), *p*H 6·2, 15 ml.; glucose 100 mg.; vitamin B_1 2–7 mg.

Method. The materials are shaken in a conical flask in a bath at *c.* 28° while O_2 passes, the vitamin B_1 being added last. After $1\frac{1}{4}$ hr. 100 mg. glucose are added, and the reaction is stopped after $2\frac{1}{2}$ hr. The flask is cooled while the O_2 stream continues, the contents are quickly centrifuged in previously cooled tubes and the remaining yeast is washed with ice-cooled water. The yeast is mixed with a minimum volume of ice-cooled water and added gradually to a convenient volume of boiling water sufficiently slowly to prevent appreciable cooling. The suspension is now centrifuged and washed with hot water. To the extract (30 ml.) after cooling 5 ml. 25 % neutral lead acetate are added and the lead precipitate is collected by centrifuging, suspended in 7–8 ml. water and treated with H_2S for 15 hr. From this stage onwards where possible cooling upon ice is used to minimize decomposition in the acid solutions. PbS is removed by centrifuging, the centrifugate filtered (volume about 6 ml.), adjusted to *c.* *p*H 5·5 by addition of NaOH and the H_2S removed by aeration. The cocarboxylase

present can be estimated approximately on 0·02 ml. by conversion into thiochrome form, though there is usually more fluorescence than cocarboxylase activity, owing presumably to the simultaneous presence of the monophospho-compound. The solution kept at 0° gradually loses activity. 0·05 ml. of the solution has about the same activity with washed yeast as some 3·5 γ Lohmann's pure cocarboxylase.

Notes. 1. The *p*H is approximately 6·5; trials at other reactions have led to no improvement.

2. The amounts have been increased to 200 g., 50 g. per flask in similar proportions, but the yield has been rather decreased.

3. The phosphorylation of about 1 mg. vitamin B_1 by 10 g. yeast is about the best so far reached.

4. The result is better if the yeast is fresh. We have not succeeded with dried yeast.

5. Special tests have not been made to decide whether the sugar addition is necessary; in many experiments amounts from 15 to 100 mg. sugar have been added initially in addition to the subsequent amount.

6. The method is based upon some 30 separate experiments.

SUMMARY

A method is described for the preparation of crude cocarboxylase from synthetic vitamin B_1.

We are grateful to the Medical Research Council for grants in aid of this work and to Messrs Bayer and Hoffmann La Roche for gifts of synthetic vitamin B_1, and to Prof. Lohmann for a specimen of pure cocarboxylase.

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XCIII. WOOD CELLULOSE

V. "TRIMETHYLATED" CELLULOSE FROM THIRIENA PULP

By DAVID JAMES BELL

From the Biochemical Laboratory, Cambridge

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Some years ago we carried out the first systematic investigation by the methylation process of the constitution of α-celluloses from several different woods [Bell, 1932, 1, 2, 3]. Employing the principles of manipulation current then, we found it impossible to methylate the celluloses above 37–39 % methoxyl, a value considerably short of the theoretically attainable maximum of 45 %. After cleavage by the procedure of Irvine & Rose [1906] under carefully controlled conditions, we were able to obtain two distinct products from the methylated celluloses (*a*) almost pure 2:3:6-trimethyl methylglucoside and (*b*) a so-called "resistant portion", thus termed because it resisted further cleavage and could not be methylated above a maximum of 32 % OMe.

As this behaviour persisted throughout the whole series of wood celluloses examined, we were forced to conclude that the presence of resistant portions in wood celluloses was a manifestation of some intramolecular condition distinguishing them from cotton. Moreover, resistance to methylation and to cleavage persisted after complete dissolution of the cellulose fibres in an acetylating medium.

Recently however Barsha & Hibbert [1936] reported a method for completing the etherification of wood cellulose by disintegrating the partially methylated fibres by dissolving in $CHCl_3$, thus presumably exposing fresh surfaces for reaction. In this way a product having over 44 % OMe was obtained. After cleavage by HCl in CH_3OH at 100–125° only a very slight residue remained, but no characterization of the cleavage products was made beyond recording boiling points. Furthermore, no description was afforded of the procedure adopted to induce solution of the fibres in $CHCl_3$, nor were the $CHCl_3$ solubility of the partly methylated material and the properties of the trimethylated product described.

We have therefore applied Barsha & Hibbert's technique to a wood cellulose previously examined, namely the so-called Thiriena pulp (a mild sulphite-bleach pulp from soft woods), and we have been able substantially to confirm the findings of the above authors with respect to our material.

As before, methylation ceased at 40 % OMe and the fibrous material was only partially soluble in chloroform at −10 to 80°. However, it was found possible by a simple process of hand-grinding with Na_2SO_4, $CHCl_3$ and glacial acetic acid to effect gradual solution of over 90 % of the material. Methylation of the solubilized material raised the OMe content above 43 % and cleavage of over 90 % resulted under conditions optimum for the isolation of the resistant portions. The residual solid (8 %) thus obtained, after precipitation from $CHCl_3$ solution by light petroleum, left a negligible residue on cleavage with fresh reagents.

In agreement with Barsha & Hibbert, we can therefore state that wood celluloses do not contain any appreciable amount of material defying complete etherification or cleavage. We desire to make the point, however, that complete identity between cotton and wood celluloses remains still an open question.

Experimental

Methylation. 3 g. Thiriena pulp were treated 8 times by Barsha & Hibbert's technique [1936]. Thoroughly washed and dried material showed OMe 39·7 %. Four further treatments were given, making a total of 12. The final yield was 2·7 g. OMe, 40·0 %.

A specimen of this fibrous material was shaken at 20° for 48 hr. with 20 parts dry $CHCl_3$, when less than 25 % of the cellulose dissolved. The residue resisted further attempts at dissolution, confirming previous findings [Bell, 1932, 1].

Almost complete solution was obtained in the following way: 2·712 g. material (OMe 40 %) were shaken for 48 hr. with a mixture of 400 ml. $CHCl_3$ and 135 ml. glacial acetic acid, when much dissolved. The gelatinous residue was separated by filtration through a 1 cm.-deep layer of anhydrous Na_2SO_4 packed on a G 3 Jena glass filter. After washing with $CHCl_3$, the insoluble residue was isolated by dissolving out the salt with hot water. The white glassy residue was ground with several g. Na_2SO_4 moistened with $CHCl_3$ and glacial acetic acid, and when well dispersed, the whole was shaken with $CHCl_3$ for 24 hr. Insoluble material was recovered as before and reground and shaken with $CHCl_3$. The same filter was used throughout to minimize mechanical loss. By this treatment the insoluble material was diminished to 0·169 g. (6·2 % of the starting material).

The united soluble fractions were isolated by distilling off the $CHCl_3$ in the presence of excess water, filtering, washing and drying. 2·458 g. were obtained (90·6 % of starting material). This was then methylated 4 times according to Barsha & Hibbert; the product was completely soluble in $CHCl_3$ and after removing a trace of precipitate by centrifuging at 14,000 r.p.m. showed $[\alpha]_D^{20°}$ 0·0° ($c=1·3$; $l=2·0$) and OMe, 43·1 %.

Cleavage. 2·21 g. material (OMe 43·1 %) were heated in a sealed tube at 80° for 400 hr. with 23 ml. CH_3OH containing 1 % dry HCl gas. The contents of the tube were then washed into centrifuge tubes, spun and the supernatant fluid collected. The residue was washed 3 times with CH_3OH, dried and weighed. It amounted to 0·18 g. (8·1 % of starting material). Unlike the original "resistant portions" it was completely soluble in $CHCl_3$, giving a brown solution which was decolorized by shaking with charcoal and filtering through $BaCO_3$. After removing the chloroform in the presence of water, the colourless dried residue (0·16 g.) was treated for 400 hr. as before with CH_3OH—HCl in a sealed tube, when only a minute trace of solid remained. The hydrolyzed material was added to the main fraction of the cleavage products.

The cleavage products. These amounted to 2·016 g. Since Haworth *et al.* [1937] have shown that treatment with acid CH_3OH seems to induce some condensation between molecules of 2:3:6-trimethyl glucose, and since the small amount of material available rendered separation by fractional distillation impracticable, the cleavage products were heated with 25 ml. 0·6 *N* HCl until a constant rotation was observed (4 hr.) and the hydrolysate worked up in the usual manner after neutralization by Ag_2CO_3. Yield, 1·75 g. colourless syrup.

1·65 g. of this material were extracted 6 times with 50 ml. amounts of dry ether. The residue weighed 0·07 g. (4·2 %) and showed OMe 37 %. The ether

extract on evaporation yielded 1·555 g. colourless syrup, $n_D^{20°}$ 1·4770, which rapidly crystallized in the manner characteristic of 2:3:6-trimethyl glucose and was identified as such by the usual methods. No evidence of isomeric trimethyl glucoses could be obtained.

SUMMARY

1. Methylation of wood cellulose to a methoxyl content of over 43 % has been effected, confirming the findings of Barsha & Hibbert. The product shows $[\alpha]_D$ 0° in $CHCl_3$, whereas trimethyl cotton shows $[\alpha]_D - 10°$.

2. Over 90 % of the trimethyl cellulose is transformed into simple glucosides by the action of CH_3OH containing 1 % HCl at 80°. The only cleavage product identifiable was 2:3:6-trimethyl glucose.

3. Previous findings by the author of a portion of wood celluloses resisting methylation and cleavage would seem to have their basis in mechanical rather than purely chemical circumstances.

We wish to acknowledge with thanks the gift of a supply of Thiriena pulp by Dr J. L. A. Macdonald, of Messrs Tullis, Russell, of Markinch, Fife; also the technical assistance given by Mr D. Dewey.

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XCIV. THE BLEACHING ACTION OF ALKALINE HYDROGEN PEROXIDE ON WOOD

By WILLIAM GEORGE CAMPBELL and GEOFFREY SWANN

From the Section of Wood Chemistry, Forest Products Research Laboratory, Princes Risborough, Aylesbury, Bucks

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It is well known that in the presence of concentrated H_2O_2 and alkali wood undergoes vigorous decomposition at ordinary temperatures. At comparatively low concentrations, however, H_2O_2 has a bleaching action on wood which has found some application commercially. The mechanism of this bleaching action is little understood, and Schorger [1926, p. 385] has stated that the light colour obtained is seldom permanent. Marggraf [1917], who apparently first studied the action, obtained, in presence of NH_3, satisfactory results with freshly felled maple, lime, aspen and white poplar woods. He observed that during the bleaching process the system rapidly became acidic. Grüss [1923] observed microscopically that after warming with concentrated H_2O_2 and subsequent drying the tracheids of pine wood became encrusted with crystalline material. Further, the treated tissue failed to give the so-called lignin colour reaction with phloroglucinol and HCl. König [1906, 1, 2] developed a method of estimating the cellulose content of forage grasses which was based upon the decomposition of lignin by means of H_2O_2 and NH_3, apparently assuming that no decomposition of cellulose took place under the conditions of the determination. That isolated alkali-lignin can be partially converted into soluble products by H_2O_2 has been shown by Anderzen & Holmberg [1923]. The present series of experiments was undertaken in order to test the bleaching effect of H_2O_2 on a variety of woods and, in three specific instances, to examine the effect in terms of current analytical methods.

Experimental

Samples (2 g., air-dry) of each of the following woods in sawdust form were mixed with 25 ml. of a solution consisting of 4 parts H_2O_2 (20 vol. AnalaR), and 1 part 11·5 % aqueous NH_3 at room temperature (20°):

Douglas fir heartwood (*Pseudotsuga taxifolia* Brit.). Jarrah heartwood (*Eucalyptus marginata* Sm.). Gaboon ebony heartwood (*Diospyros? crassifolia*). Bog oak (*Quercus* sp.). Purpleheart (*Peltogyne porphyrocardia* Griseb.). Barwood (*Pterocarpus tinctoreus* Welw.). Honduras rosewood (*Dalbergia stevensonii* Standl.). Andaman padauk (*Pterocarpus dalbergioides* Roxb.).

With the exception of purpleheart and ebony all of the samples were bleached to pale yellow or buff shades after 24 hr. The sample of purpleheart required 50 ml. of bleaching solution and 48 hr. to bring it to an ivory yellow shade, whilst the ebony required 75 ml. of solution and 72 hr. to produce a buff-coloured residue which was completely macerated and only represented a small proportion of the starting material. After thorough washing with cold water and drying *in vacuo* at 35–40° none of the bleached woods darkened and no appreciable changes in colour have been observed to take place on subsequent exposure to air and light for 12 months. Drying of bleached wood at 100–105° invariably caused darkening in colour which was more accentuated in some species than in others.

2 g. (air-dry) samples of each of the following woods in sawdust form were mixed with 25 ml. of a freshly prepared bleaching solution consisting of 5 parts H_2O_2 (20 vol. AnalaR) and 1 part 10 % NaOH at room temperature (20°):

Tasmanian oak (*Eucalyptus obliqua* L'Hérit.). Tasmanian oak or Alpine ash (*Eucalyptus delegatensis* R. T. Baker). Tasmanian oak or Australian oak (*Eucalyptus regnans* F. Muell.). Oak heartwood (*Quercus* sp.). Beech (*Fagus sylvatica* L.). Pine (*Pinus sylvestris* L.). Hard maple (*Acer saccharum* Marsh). Ash (*Fraxinus excelsior* L.).

After 24 hr. the samples of the three eucalypts as well as those of maple and ash had become bleached to a pure glistening white colour while the samples of the remaining species were bleached to a white colour with a faint yellowish tinge. In the case of *Eucalyptus obliqua* the concentration of NaOH (*c.* 2 %) could be reduced to 1 % provided that 40 ml. of bleaching solution were used for every 2 g. sawdust. The samples of bleached wood in all cases remained white provided that they were thoroughly washed with cold water and dried either at 40° *in vacuo* or by washing with 95 % alcohol followed by ether. Drying at 105° induced darkening to a pale yellow colour in each case. The surface layers of solid commercial samples of Tasmanian oak (*Eucalyptus* sp.) were bleached to a very light colour in approximately 1 hr. after being rubbed over in turn with H_2O_2 (10 vol.) and either dilute NaOH or NH_3.

The effect of alkaline H_2O_2 on the chemical composition of wood

(1) *Douglas fir.* A 60 g. (air-dry) sample of 60–80 mesh Douglas fir wood flour was mixed with 600 ml. 20 vol. H_2O_2 and kept at 20° for 24 hr. after adding 120 ml. 11·7 % aqueous NH_3. A 100 g. sample of the same material was treated for six successive periods of 48 hr. each as follows. For the first treatment the wood was mixed with 1 l. H_2O_2 and 100 ml. 11·7 % NH_3. After 48 hr. the liquor was filtered off and for each subsequent treatment a further charge of 500 ml. H_2O_2 and 100 ml. NH_3 was added. After bleaching both samples were thoroughly washed with warm water until neutral to litmus, partially dried at 40° *in vacuo* and analysed (Table I).

Table I. *Analysis of 60–80 mesh Douglas fir and jarrah woods before and after bleaching with H_2O_2 and NH_3 at 20°*

(Results expressed as % by wt. of oven-dry original wood.)

	Douglas fir			Jarrah	
	Original wood	After bleaching for 24 hr.	After bleaching for 288 hr.	Original wood	After bleaching for 24 hr.
Loss in wt.	—	11·58	19·97	—	18·53
Cold water-soluble	3·48	0·19	0·67	0·49	0·58
Hot water-soluble	5·60	1·17	2·80	1·40	2·29
1 % NaOH-soluble	16·85	11·93	15·19	21·47	13·28
C. and B. cellulose	58·43	57·06	53·62	50·10	49·75
α-Cellulose	40·60	38·99	32·82	40·93	40·74
α-Cellulose as % of C. and B. cellulose	69·48	68·32	61·21	81·70	81·90
Lignin	26·94	22·29	17·79	36·98*	21·43*
Total pentosans	8·27	7·06	4·72	13·76	12·39
Pentosans in cellulose	3·18	2·81	2·08	5·86	5·73
Pentosans not in cellulose	5·09	4·25	2·64	7·90	6·66
Total methoxyl	4·48	3·77	2·92	7·13	5·55
Methoxyl in lignin	4·05	3·44	2·53	6·09	4·53
Total CO_2 on hydrolysis with 12 % HCl	0·605	0·67	0·492	1·032	0·985
CO_2 in cellulose on hydrolysis with 12 % HCl	0·362	0·28	0·229	0·422	0·405

* Uncorrected for "kino".

60 g. of 60–80 mesh, air-dry jarrah wood were treated under the same conditions for 24 hr. using 600 ml. 20 vol. H_2O_2 and 120 ml. 11·7 % NH_3. The residue was analysed after washing and drying as above (Table I).

40 g. of 60–80 mesh, air-dry Tasmanian oak (*Eucalyptus obliqua*) mixed with 600 ml. 20 vol. H_2O_2 and 120 ml. of 10 % NaOH were well stirred and kept at 20° for 24 hr. The wood residue which had become pure white in colour was collected and washed in the usual way. Before analysis (Table II) excess water was removed by washing with alcohol followed by ether.

Table II. *Analysis of Tasmanian oak* (Eucalyptus obliqua) *before and after bleaching with H_2O_2 and NaOH for 24 hr. at 20°*

(Results expressed as % by wt. of original oven-dry wood.)

	Original wood	White residue after bleaching
Loss in wt.	—	23·96
Hot water-soluble	7·44	1·92
1 % NaOH-soluble	18·52	10·71
C. and B. cellulose	58·86	54·50
α-Cellulose	46·60	32·99
α-Cellulose as % of C. and B. cellulose	79·17	60·45
Lignin	19·57*	12·64*
Total pentosans	16·54	9·75
Pentosans in cellulose	9·33	6·51
Pentosans not in cellulose	7·21	3·24
Total methoxyl	6·62	4·46
Methoxyl in lignin	4·44	3·03
Total CO_2 on hydrolysis with 12 % HCl	0·815	0·870
CO_2 in cellulose on hydrolysis with 12 % HCl	0·590	0·427

* Uncorrected for "kino".

The action of alkaline hydrogen peroxide on isolated lignin

2·6144 g. of oven-dried (105°) beech lignin isolated by the method of Bamford & Campbell [1936] were finely powdered and mixed at room temperature with 200 ml. 20 vol. H_2O_2 and 20 ml. 2*N* NaOH. After 42 hr. a further 50 ml. H_2O_2 were added and after 90 hr. the dark brown solution was filtered on an alundum crucible of porosity RA 360. A small insoluble residue (0·0527 g.) was obtained after washing and drying at 105°. The addition of a slight excess of $BaCl_2$ to the clear, brown filtrate gave rise to a bulky buff-coloured precipitate which, after dehydrating in several changes of alcohol, centrifuging and drying *in vacuo* at 40°, was recovered as a pale brown powder which was not further investigated.

Discussion of results

The results show that, irrespective of whether they are in the seasoned or unseasoned condition, a wide range of woods can be bleached to permanent, light shades of colour by means of dilute alkaline H_2O_2. Provided that the desired degree of bleaching can be obtained in a short period of time very little deterioration of wood substance is likely to take place, and dilute H_2O_2 and NH_3, for example, should be suitable for bleaching the surfaces of veneers and speciality wood products. In most of the samples of sawdust examined bleaching was complete after 24 hr., but it has been shown that purely surface bleaching can take place in a much shorter period. Prolonged contact with the reagents merely leads to marked degradation of the major wood components without any improvement in bleaching effect. This is illustrated by the data in Table I. In the

case of Douglas fir, for instance, the loss in wt. incurred after bleaching for 24 hr. is largely accounted for by depletions of minor components and lignin. The loss of Cross and Bevan cellulose is slight, but its significance is confirmed by the results for α-cellulose, pentosans and CO_2 in the cellulose, all of which are slightly lower in the bleached wood. For most purposes this small amount of cellulose degradation is probably not serious. The remaining data for Douglas fir (Table I) show that, over a prolonged period, dilute H_2O_2 and NH_3 can cause extensive decomposition of all the major wood components at room temperature. The bleaching of the dark brown jarrah wood to cream colour was found to proceed smoothly. The data in Table I show that the loss in wt. in this case is largely made up of material derived from the apparent lignin. That this material is what is commonly referred to as "kino" is strongly suggested by the fact that a sample of exuded "kino" from a gum pocket in a jarrah tree [Campbell & Bryant, 1937] was transformed by H_2O_2 and NH_3 into soluble decomposition products of a pale straw colour. Under the conditions stated jarrah cellulose suffers little or no degradation during the bleaching process.

The most striking colour changes observed in the present study were those which occurred when NaOH was used as the activating agent for the H_2O_2. The observation was first made on a sample of Tasmanian oak (*Eucalyptus obliqua*) in conjunction with Dr W. E. Cohen to whom grateful acknowledgement is here made. The bleached wood was pure white. It is shown above that similar results are obtainable with other hardwoods but a pure white product was not obtained from either of the softwoods examined. The data in Table II show that, in order to obtain a pure white residue, it is not necessary to remove all of the lignin from wood. The bleached wood gave a red chlorine:sodium sulphite colour reaction which was more pronounced than that given by the original wood. Further experiments are envisaged in which it is proposed to investigate more closely the properties of this white product. Apart from considerations of expense there are already indications that its production on a large scale from sawdust, groundwood etc., is unlikely to proceed as smoothly as its production under purely laboratory conditions, but further investigation of these latter questions could well be delayed until more fundamental information concerning the product itself is available.

The above experimental data go at least some way towards explaining the little understood mechanism of the bleaching action. The question naturally arises as to whether a dual role is played by the alkali in the system. It has been found [Forest Products Research Board, 1935] that the action of aqueous NH_3 in a wide range of concentrations on wood at room temperature is mainly confined to the solution of minor components with only slight decomposition of lignin and furfuraldehyde-yielding complexes and no decomposition of cellulose. Dilute NaOH at room temperature is known to be capable of removing hemicelluloses, some lignin and minor components from wood. In addition it probably induces slight decomposition of cellulose. Little or no bleaching action results from the contact of wood with dilute alkali alone. The immediate effect of adding either NH_3 or NaOH to wood is a pronounced darkening in colour. This is no doubt due in part to the extraction of certain components from the wood and in part to chemical reaction between those components and the alkali. There can be little doubt that although the most important function of the alkali is the liberation of energy from the H_2O_2 much must also depend on its solvent and swelling properties. In the bleaching process the order of events is usually as follows: (1) darkening of the wood followed by the leaching out of a dark-coloured extract, (2) simultaneous bleaching of wood and extract with

evolution of gas. In no case did the extract finally become colourless nor did the wood assume its palest shade of colour until after it had been thoroughly washed with water. Since the carbohydrates in wood are probably all white it must be assumed that bleaching of the wood as a whole is the result of leaching and chemical degradation of coloured minor components and/or lignin. It must be assumed that the non-carbohydrate components are decomposed by oxidation and that the products of the oxidation, either alone or in combination with alkali, are soluble in water. This view is strengthened by the knowledge [Anderzen & Holmberg, 1923] that lignin is decomposed to a mixture of carboxylic acids under conditions similar to those obtaining during the bleaching of wood.

It is a matter for comment that the apparent lignin which remains in certain woods after bleaching is colourless. This may be taken to indicate either that the original lignin present in such woods as Tasmanian oak, European beech, ash, oak etc., is colourless, or that in the course of the bleaching process lignin is oxidized in such a way that the groups which are responsible for colour are removed. In this connexion it was observed that the white residue from all the woods mentioned above only gave a very faint pink colour reaction with phloroglucinol:HCl in contrast to the deep magenta colour reactions of the original woods. The corresponding colour reactions given by the bleached softwoods examined were much more pronounced than those given by the bleached hardwoods. Without exception the lignin residues obtained in the analysis of the bleached woods were of the normal, chocolate brown colour. We are indebted to our colleague, Mr S. H. Clarke, for permission to state that microscopical examination of sections of Scots pine and ash woods which had been treated with 20 vol. H_2O_2 and NH_3 revealed that lignin had been removed from both middle lamella region and secondary walls. Further, it was concluded that the somewhat feeble colour reactions given by the secondary walls of bleached ash wood with chlor-zinc-iodine were an indication that the cellulose present in those walls was still masked by another substance.

If, during the bleaching processes referred to, conditions are sufficiently severe there is significant action on the wood cellulose. This is apparent from the results for Douglas fir (Table I) and Tasmanian oak (Table II) which indicate that the total effect is not merely confined to a small loss in Cross and Bevan cellulose. On the contrary it is much more insidious in that the % α-cellulose which can be recovered from the bleached woods are much lower than in the corresponding original woods. The full significance of these results will be discussed elsewhere but, in view of the uncertainty expressed by Heuser *et al.* [1924, p. 118] and by Schorger [1926, p. 270] concerning the action of H_2O_2 on cellulose, it should be mentioned that they are consistent with the view that the cellulose has undergone hydrolysis and not oxidation as might have been expected. This view is confirmed in part by the observation that the yield of CO_2 on hydrolysis of the Cross and Bevan cellulose is not enhanced as a result of the bleaching process, but chiefly by a previous observation made in this Laboratory [Campbell, 1937] that, under certain conditions, acid hydrolysis can produce the same effect on Cross and Bevan cellulose as has been observed here.

Summary

1. It has been shown that a wide range of woods can be bleached to light shades of colour by H_2O_2 (20 vol.) in the presence of NH_3 or dilute NaOH at room temperature.

2. The residue remaining after treating certain hardwoods at room temperature with H_2O_2 (20 vol.) and 2 % NaOH is pure white although it retains a considerable amount of apparent lignin.

3. The colour of bleached wood is reasonably permanent provided that drying of the finished product is carried out at a low temperature (*c.* 40°).

4. The order of events in the bleaching process is (1) darkening of the wood followed by the leaching out of a dark-coloured extract and (2) simultaneous bleaching of extract and wood with evolution of gas.

5. It is suggested that certain non-carbohydrate components of the wood are leached out and decomposed by oxidation into products which, either alone or in combination with alkali, are soluble in water.

6. Under sufficiently severe conditions there is during bleaching some degradation of cellulose which is shown to be of a hydrolytic nature.

The authors are indebted to Mr W. A. Robertson, Director of Forest Products Research, for permission to communicate these results.

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XCV. STUDIES ON THE VITAMIN B COMPLEX

I. THE OCCURRENCE OF THE RAT ANTI-DERMATITIS FACTOR (VITAMIN B_6) IN FISH AND FISH PRODUCTS

By GULBRAND LUNDE and HANS KRINGSTAD

From Hermetikkindustriens Laboratorium, Stavanger
(Research Laboratory of the Norwegian Canning Industry)

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It was first shown by Goldberger *et al.* [1926] that the vitamin B complex consisted of two factors, the anti-beriberi factor and a factor which was necessary for growth and prevented a pellagra-like dermatitis in rats. In Goldberger's opinion this factor was identical with a vitamin which prevented pellagra in human beings and was called P-P (pellagra-preventing) factor. This factor was afterwards called vitamin G. It was also assumed by Goldberger *et al.* [1928] that this factor was identical with a factor which prevented black-tongue in dogs and rabbits. Later investigations showed, however, that it was difficult to produce the pellagra-like dermatitis regularly in rats. The vitamin G content of foods was therefore determined biologically, not by its pellagra-preventing effect, but by its stimulating effect on growth. Aykroyd [1930] later demonstrated that this vitamin G or B_2, as it was named by English authors, was present in foods which were constituents of typical pellagra-producing diets. This discrepancy was explained by the finding that vitamin G (or B_2) consisted of two components, lactoflavin and a further supplementary factor [György *et al.* 1933]. Several workers demonstrated later that lactoflavin could not cure the pellagra-like dermatitis in rats [György, 1935; Chick *et al.* 1935; Harris, 1935]. The rat dermatitis-preventing factor was termed vitamin B_6 by György. Later Birch *et al.* [1935] and Dann [1936] demonstrated that the P-P factor was neither lactoflavin nor vitamin B_6. Vitamin B_6 was found to differ from the P-P factor in chicks [Lepkovsky *et al.* 1936]. The distribution of vitamin B_6 was studied by Birch & György [1936] who found that fish and wheat germ were good sources.

The present paper deals with the occurrence of vitamin B_6 in different fish and fish products.

As a standard of reference we used Peters' eluate, placed at our disposal by the I. G. Farbenindustrie A. G., Wuppertal/Elberfeld. 0·2 ml. of this eluate was sufficient to cure dermatitis in rats. Peters' eluate was also prepared in this laboratory according to the original description by Kinnersley *et al.* [1933]. 1 ml. of this preparation corresponded to 10 g. baker's yeast and 0·5 ml. cured dermatitis in rats.

We used in these experiments the diet described by György [1935]. Our second method of purifying the casein was that used by Elvehjem *et al.* [1936] in their work on pellagra.

Experimental

Young rats weighing 40–50 g. and about 4 weeks old were placed on the following diet:

	%
Casein, purified	18
Rice starch	68
Butter fat	9
Salt mixture (Osborne & Mendel)	4
Cod liver oil	2

In addition the rats received daily 6γ vitamin B_1 (cryst. Hoffmann-La Roche) and 10γ synthetic lactoflavin (Hoffman-La Roche). Some animals received 2 ml. raw egg-white daily as a source of lactoflavin.

Purification of the casein. In the first experiment we extracted the casein with 70 % alcohol. Later the casein was dissolved in NH_4OH and precipitated with HCl.

1st method. 3 kg. casein (techn.) were extracted with *c.* 7 l. hot 70 % ethyl alcohol for ½ hr. After filtration the extraction was repeated with fresh alcohol. This procedure was repeated until the extract showed no yellow fluorescence when examined in ultraviolet light (lactoflavin-free). The casein was dried at 45–50° and ground.

2nd method. 10 kg. casein (techn.) were dissolved in *c.* 170 l. H_2O with 750 ml. NH_4OH (sp. gr. 0·88). The solution was acidified with HCl (pH = 4·5). The precipitated casein was filtered through gauze and the procedure was repeated three times. The final product was dried at 45–50° and ground to a fine powder.

The rats were kept in separate cages with a screen bottom to prevent coprophagy. The rats were weighed twice a week.

On this diet growth ceased after 2–4 weeks and some of the rats lost weight. The first sign of dermatitis developed after 3½ weeks in some of the rats. After 4 weeks 57 % of the rats showed dermatitis. After 6½ weeks the dermatitis developed in all rats to a greater or less degree.

The rats received the substance to be tested in addition to the basal diet when the symptoms of dermatitis were pronounced. Usually the dermatitis increased in severity during the first days of treatment when the substances tested were low in vitamin B_6, and in cases with very severe dermatitis no sign of recovery could be observed until after 2–3 weeks.

The dermatitis developed as severely and rapidly in rats receiving pure lactoflavin as in those receiving raw egg-white as a source of lactoflavin. As several authors have described an egg-white syndrome in rats, this result is of special interest, and will be dealt with in a subsequent paper.

Results

A summary of the results is given in Table I. It was found that the time necessary for complete healing of the dermatitis varied according to the severity of the original condition.

Varying amounts of anti-dermatitis factor were found in the products tested. Cod liver was a very rich source, 0·25 g. a day being sufficient to cure even a severe dermatitis. Thus cod liver is at least as rich in vitamin B_6 as Peters' eluate, and may prove a better source for preparing concentrates than baker's yeast. It is of interest to note that cod flesh was not as rich in the factor as flounder.

Table I. *Rat anti-dermatitis factor (vitamin B_6) in various products*

Product tested	Amount fed g.	No. rats	Time for healing of dermatitis weeks	Av. gain in wt., g. in 4 weeks	Av. gain in wt., g. in 6 weeks
Peters' eluate	0·4 ml.	1	$3\frac{1}{2}$	29	49
	0·2 ml.	3	3–6	18	29
Cod liver, raw	1·0	3	3–$3\frac{1}{2}$	40	57
	0·5	3	2–4	25	38
	0·25	4	2–6	24	28
Cod roe, canned	1·5	1	3	33	—
	1·0	2	1 no improvement in $3\frac{1}{2}$ weeks; 1 cured in 3 weeks	17* 32	— 38
	0·5	3	3–4	30	44
Cod flesh, raw	0·5	4	1 died after 4 weeks; 3 no improvement after $2\frac{1}{2}$ weeks	— 0†	— —
	2·0	3	2–4	10	—
Flounder flesh, raw	2·0	2	$2\frac{1}{2}$	40	56
	1·0	2	3–4	33	—
Herring milt, canned	1·0	2	Not cured in 5 weeks	17	—
Kippered herrings, canned	1·0	4	4–6	21	31
Herring sardines, canned	1·0	4	3–5	26	38
Brisling sardines, canned	2·0	1	4	34	—
	1·0	2	2–6	23	39
Crab, canned	0·5	4	2 died after $2\frac{1}{2}$ weeks; 2 no improvement after 3 weeks	−13† 7	— —
	2·0	2	$3\frac{1}{2}$	17	—

* In $3\frac{1}{2}$ weeks. † In $2\frac{1}{2}$ weeks.

As the canned products tested were effective in curing the dermatitis, it must be concluded that canning does not destroy the factor.

It will be seen from Table I that some of the products tested had a greater growth-promoting effect than others. Thus growth did not occur when crab and cod flesh were added to the basal diet, though the dermatitis was cured. On the other hand, the animals grew when herring milt was added, but the dermatitis was not cured. There would seem to be at least two factors necessary for normal development of the rat, an anti-dermatitis factor and a growth factor, in addition to vitamins B_1 and B_2 (lactoflavin). Edgar & Macrae [1937] found two factors, an eluate and a filtrate factor, necessary for the growth of rats in addition to vitamins B_1 and B_2. Lepkovsky *et al.* [1936] also found two factors, which seem to be identical with the factors described by Edgar & Macrae. Further studies of the occurrence and distribution of these two factors will be reported in a subsequent paper.

Summary

Several fish products were tested as sources of the rat anti-dermatitis factor (vitamin B_6). Cod liver was found to be a very good source.

The authors are indebted to Messrs Hoffmann-La Roche et Cie, Basel, for furnishing us with a sample of pure synthetic vitamin B_1.

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XCVI. STUDIES ON THE VITAMIN B COMPLEX
II. EXPERIMENTS ON THE EFFECT OF *ISO*LEUCINE ON THE "PELLAGRA-LIKE" DERMATITIS IN RATS

By GULBRAND LUNDE and HANS KRINGSTAD

From Hermetikkindustriens Laboratorium, Stavanger
(Research Laboratory of the Norwegian Canning Industry)

(Received 8 February 1938)

Sjollema [1937] reported that rats placed on a dermatitis-producing diet could be cured by daily doses of 60–90 mg. *iso*leucine. If this is true the "pellagra-like" dermatitis in rats would seem to be caused by deficiency of an amino-acid and not by avitaminosis. The diet used by Sjollema was very similar to that used in our experiments [Lunde & Kringstad, 1938]. It consisted of: glaxo casein 18·5 %, rice-starch 64 %, butterfat 8·3 %, cod liver oil 2 %, agar 2 % and salt mixture 5·2 %.

In addition to this diet the animals received each day 10γ vitamin B_1 and in some groups the same amount of lactoflavin. On this diet the rats developed dermatitis in 8–10 weeks, i.e. much later than our rats. This is probably due to the fact that the glaxo casein used in Sjollema's experiments was not further purified and contained small amounts of the antidermatitis factor.

Experimental

Young rats weighing 40–60 g. and about 4 weeks old were placed on the dermatitis-producing diet previously described [Lunde & Kringstad, 1938]. The first sign of the "pellagra-like" dermatitis occurred after 3–6 weeks. In all animals growth ceased after 2 weeks and the weights remained constant or decreased. When the dermatitis was pronounced 7 rats were given 50 mg. *d-iso*leucine (Hoffmann-La Roche) in addition to the diet. No improvement occurred. In 2 rats the amount of *d-iso*leucine was raised after 1 week to 70 mg. Four of the rats, including those receiving 70 mg., died after 2–5 weeks. In two of the remaining rats the symptoms of dermatitis increased rapidly. After 3 weeks the administration of *d-iso*leucine was stopped and the 2 rats received 150 mg. rice polish concentrate (Labco) in addition to the basal diet. Improvement could be observed after 1 week; the weights increased rapidly and the rats were completely cured in 3 weeks. The average gain in weight was 52 g. in 3 weeks.

Three other rats with dermatitis received 100 mg. *dl-iso*leucine a day for 4–5 weeks without any sign of recovery or gain in weight. The *iso*leucine was dissolved in water and given by means of a stomach tube.

Discussion

Our experiments do not confirm the finding by Sjollema that the "pellagra-like" dermatitis in rats can be cured by *iso*leucine. Sjollema does not mention which optical isomeride of *iso*leucine was used in his experiments. We used both *d-iso*leucine and a mixture of the two optical isomerides.

If *iso*leucine should be the active principle of "vitamin B_6" it must be contained in Peters' eluate, 0·2 ml. of which cures the dermatitis rapidly. Peters' eluate is prepared by a method involving the use of lead acetate which precipitates all proteins. The nitrogen content of Peters' eluate was determined. It contained 5·5 mg. per ml. calculated as *iso*leucine. This result also indicates that the "pellagra-like" dermatitis in rats cannot be due to lack of *iso*leucine in the diet.

SUMMARY

The "pellagra-like" dermatitis in rats is not due to a lack of *iso*leucine in the diet. The dermatitis was not cured by daily doses of 50–70 mg. *d-iso*leucine or 100 mg. *dl-iso*leucine.

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XCVII. ELECTROPHORESIS OF HUMAN BLOOD PLASMA

ELECTROPHORETIC PROPERTIES OF FIBRINOGEN

By EINAR STENHAGEN

From the Institute of Medical Chemistry, University of Upsala

(*Received 14 February 1938*)

Tiselius [1937, 1, 2] has described a new electrophoresis apparatus and its application to the analysis of the protein fractions in native horse serum. The new apparatus makes disturbance-free electrophoresis possible and components having different mobilities can be isolated. An important advantage of the apparatus is that the migrating protein boundaries are rendered directly visible by means of the Toepler "Schlieren" method.

The analysis of human blood plasma has now been undertaken, with the Tiselius apparatus. In the case of plasma one expects to find at least one more component than in serum, i.e. the fibrinogen. The statements in the literature regarding the electrochemical properties of this plasma constituent disagree widely [*v.* e.g. Mainzer, 1932]. Isoelectric points between *p*H 3·9 [Farkas & Groak, 1929] and *p*H 12·5 [Rossier, 1928] have been given. Kylin & Poulsen [1936] found three different fibrinogens, with isoelectric points at *p*H 5·4, 8·5 and about 12·4 respectively. The reasons for these discrepancies have been discussed by Kylin & Poulsen [1936] and others, and seem to lie mainly in differences with regard to the method used, the electrolyte milieu and the preparation of the fibrinogen.

The fibrinogen seems to be more sensitive towards thermal and chemical treatment than most other proteins. Even freezing, which has been found by Tiselius to produce no change in the electrophoretic properties of horse serum proteins, changes human blood plasma. After freezing there appear thick, cloudy precipitates and the normal electrophoretic diagram is not obtained. All plasmas tried behaved similarly in this respect. Human serum does not change its properties even after repeated freezing and there very seldom appear any precipitates. (It is necessary to draw the blood from fasting persons otherwise the colloidal fatty particles appearing in plasma after food intake may render the optical measurements impossible.)

For the reasons mentioned, only fresh oxalated plasma was used. The blood cells were centrifuged off immediately after the blood was drawn. For the serum experiments, the blood was left for about half an hour before centrifuging, in order that spontaneous clotting and subsequent contraction of the fibrin could take place. For mobility studies plasma and serum respectively were diluted four times with the buffer to be used and then dialysed overnight at 0° against a large volume of buffer solution. Acetate and phosphate buffers of constant ionic strength 0·1 were used.

The electrophoresis was carried out at 0° with a potential gradient of about 7 volt/cm. Rubber stoppers were fitted in the open ends of the electrode vessels

to reduce their areas as much as possible in order to counteract hydrostatic displacement, and all other conditions for disturbance-free electrophoresis were maintained. To make all boundaries visible from the beginning of the experiment, before switching on the current the plasma-buffer boundary was moved forward a short distance by means of a very slow injection of buffer solution into one of the electrode vessels. A slowly migrating boundary might otherwise remain hidden behind the cell end-plates during a whole run.

In horse serum Tiselius found, besides the albumin, three cataphoretically different globulins, called α, β and γ respectively. On the positive side there also appeared a fifth boundary, called δ, of almost zero mobility, which was observed in undiluted as well as in four times diluted horse sera. As all attempts to isolate the corresponding protein component failed, it was thought to represent some sort of equilibrium compound. The appearance of the δ boundary, however, has now been found to be due mainly to a conductivity disturbance which has often been observed in the electrophoresis of concentrated solutions [Tiselius & Svensson, 1938].

The electrophoretic diagram of human serum at *p*H 7·91 is seen in Fig. 1. Four different boundaries are visible, symmetrical on both sides. These boundaries correspond to albumin, globulins α, β and γ respectively. In four times diluted human serum no δ-boundary is visible, although it appears in undiluted or twice diluted serum.

Fig. 1.

Fig. 2.

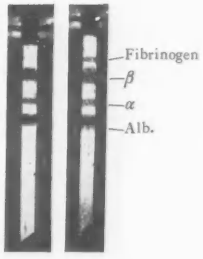

Fig. 3.

The photographs in Fig. 3 were taken after migration for 44 min. at 7·9 volt/cm.

The plasma diagram obtained under the same conditions is shown in Fig. 2. The plasma and serum from which the diagrams are obtained, were taken at the same time from one person, dialysed against the same buffer and subjected to electrophoresis under as identical conditions as possible. In the plasma diagram there appears on both sides, between the β and γ boundaries, a new boundary. As the diagrams are otherwise exactly similar and as, in addition, the electrophoretic mobilities of albumin and globulins α, β and γ respectively are found to be, within the limits of the experimental errors, identical in plasma and serum, the new boundary must doutless be due to the fibrinogen. (In order to bring out all components in the photographs, the Schlieren diaphragm has to be set in an intermediate position, hence the photographs do not show the maximum

boundary sharpness obtained with the screen in optimum position for a single boundary.) In Fig. 3 plasma and serum diagrams are placed side by side. Plasma shows the fibrinogen behind the β boundary. In this figure globulin γ is not visible, being hidden behind the end plates of the cell. In all photographs, the migration direction is downwards.

The electrophoretic mobilities of the plasma and serum components as a function of *p*H are plotted in Fig. 4. Each point is the mean of several experiments on serum and plasma. Only sera and plasma from apparently healthy persons were used. The mobility data are also given in Table I.

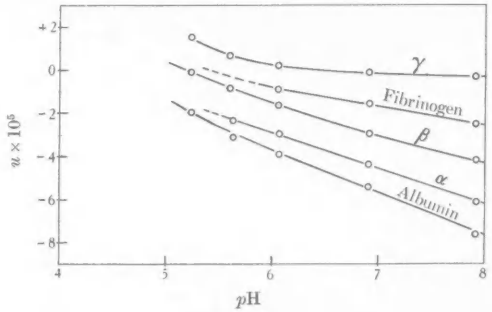

Fig. 4.

The isoelectric points given in Table I are very approximate only. In the mixtures of proteins, the isoelectric points determined cataphoretically differ somewhat from those found with the isolated protein, owing to mutual interaction or other factors.

Table I

Electrophoretic mobilities, in cm.^{2} V^{-1} sec.$^{-1}$ $\times 10^5$, of the protein components in normal human plasma and serum at different *p*H. Ionic strength 0·1. Temperature $\pm 0{\cdot}0°$.

Buffer	*p*H	Albumin	Globulin α	Globulin β	Fibrinogen	Globulin γ
Acetate	5·2	−2·0	—	−0·1	—	+1·5
Phosphate	5·6	−3·2	−2·4	−0·9	—	+0·5
,,	6·1	−3·9	−3·0	−1·7	−0·9	+0·2
,,	6·9	−5·5	−4·4	−3·0	−1·5	−0·1
,,	8·0	−7·7	−6·1	−4·2	−2·5	−0·4
Isoelectric points *p*H	—	—	(4·8)	5·2	5·4	6·4

Under the experimental conditions employed, no separation of globulin α from the albumin, below *p*H 5·5, could be detected with the optical arrangement used. In general, globulin α migrates more closely with albumin in human plasma than it does in horse serum; furthermore, its concentration in human serum is very low, being roughly about 0·25 % in concentrated serum (in horse serum about 0·95 %). The globulin β boundary is always associated with a yellow-brown colour and a slight opalescence, which shows up against the yellow colour of the albumin (due to bilirubin [Bennhold, 1932; Pedersen & Waldenström, 1937]. In plasma the globulin β boundary always tends to spread out towards the fibrinogen boundary. Below *p*H 5·7 the fibrinogen boundary does not separate from the β boundary, which appears broad and rather diffuse.

The globulin γ is always colourless. The slow migration of this component at a more alkaline reaction, as compared with horse serum, may possibly be due to a slight δ-effect. On account of the low concentration of globulin α and fibrinogen in plasma, no attempt was made to isolate all components with the rather small cells at present available. It is hoped to make use of a larger cell in the near future. Therefore albumin only was isolated, the mobility curve of which is given in Fig. 5.[1] The isoelectric point is at *p*H 4·64, which is the same as found by Tiselius for horse serum albumin.

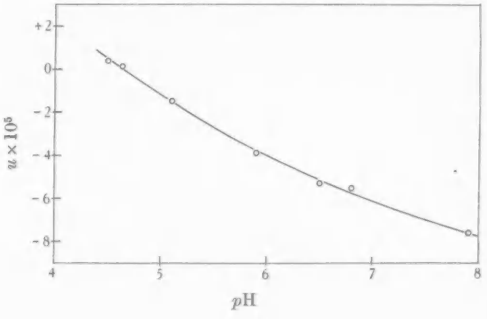

Fig. 5.

The isoelectric point of the fibrinogen in the protein mixture of plasma lies in the region of *p*H 5·2–5·6, its exact value cannot be obtained from these experiments for the reasons stated above. The approximate value arrived at is in agreement with that found by Nordbö [1927], who used the potentiometric titration method. As Kylin & Poulsen claim to have found by means of electrophoresis two fibrinogen fractions with isoelectric points more alkaline than *p*H 8, a special experiment was carried out with concentrated plasma to test this, as there is the possibility that a component present only in a very low concentration in native plasma may escape detection in four times diluted plasma. (With the experimental arrangement employed the lowest concentration in which a protein can be detected optically is about 0·03–0·05 %.) Undiluted plasma was subjected to prolonged electrophoresis (5 hr. at 4 volt/cm.). At *p*H 7·5 no cathodic migration was observed. As a further test, the solution behind the slowest boundary was isolated and tested with salicylsulphonic acid. No trace of protein could be found. The claim of Kylin & Poulsen must be considered in the light of the fact that in their experiments the conditions for disturbance-free electrophoresis were not maintained (undiluted plasma, unbuffered solutions, long runs at a relatively high temperature etc.).

An approximately quantitative estimation of fibrinogen in plasma gives a value in good agreement with those ordinarily found under normal conditions, i.e. 0·2–0·4 %.

Summary

Human blood plasma and serum have been subjected to electrophoretic analysis by means of the new apparatus of Tiselius. Serum was found to contain, in addition to the albumin, three cataphoretically different globulins,

[1] An analysis of this albumin in the ultracentrifuge, kindly performed by Dr K. Pedersen of the Institute of Physical Chemistry, Upsala, also showed it to be free from globulin.

corresponding to those found by Tiselius in horse serum and called α, β and γ respectively. Plasma, besides the components mentioned, contains one more component, the fibrinogen. This was found to migrate more slowly than β globulin but faster than γ globulin and to be an electrophoretically well-defined protein having its isoelectric point at about *p*H 5·4. The electrophoretic mobilities of the plasma and serum proteins over the *p*H range 5–8 are given, together with the approximate isoelectric points. The isoelectric point of electrophoretically isolated serum albumin was found to be at *p*H 4·64. Some earlier results by other investigators are discussed.

The author wishes to express his thanks to Dr Tiselius for valuable discussions. Thanks are also due to Miss Gun Gustbée for valuable assistance.

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XCVIII. THE MANOMETRIC DETERMINATION OF AMINO-ACIDS

By MORTON F. MASON

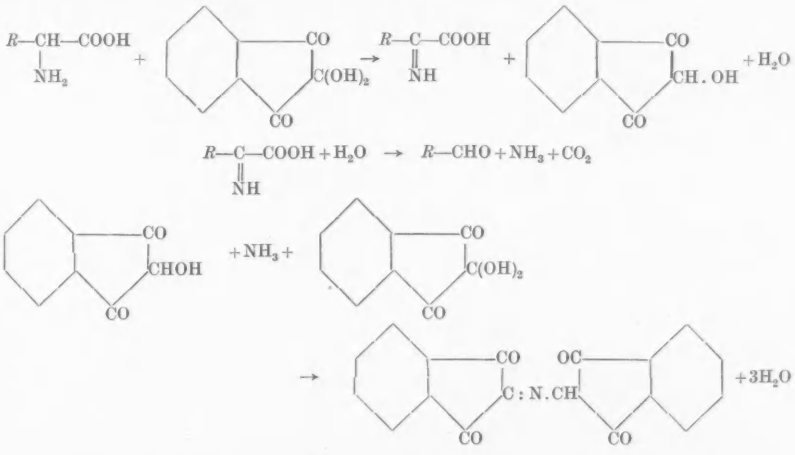
From the Physiological Laboratory, Cambridge

(*Received 7 February 1938*)

In a recent investigation dealing with the locus of deamination [Mason, 1937] the author estimated amino-acids by a method (not described) involving manometric measurement of the CO_2 split from the carboxyl groups of those acids by heating with ninhydrin, a procedure previously suggested as feasible by Van Slyke & Dillon [1936]. The validity of the gasometric technique developed was established by analyses of many amino-acids and related compounds. It is the purpose of this communication to describe the method employed and to give the results of these tests.

More recently Van Slyke & Dillon [1938] have given a detailed account of the gasometric procedure as they have developed it. The technique which they recommend differs only slightly from that used in obtaining the data submitted in this paper.

The reaction between α-amino-acids and ninhydrin in aqueous solution appears to proceed in a manner analogous to the formation of murexide and is to be formulated as follows [Ruhemann, 1911]:



The cyclic acids proline and hydroxyproline undergo a somewhat different condensation with ninhydrin, in which, however, they are decarboxylated [Grassmann & van Arnim, 1934]. The configurations which are able to participate in the reaction appear to be sharply limited, in so far as the evolution of CO_2 is concerned. They include those substances having a free NH_2 group in the α position adjacent to a carboxyl and certain acids with cyclic imino groups in

positions similar to those of the prolines. The non-reactivity of *N*-methyltyrosine (surinamine) is an example contrary to the generalization that all substances with the structure $R{CH_2}.NH.CH(R).COOH$ react [Van Slyke & Dillon, 1938]. Those acids in which the amino group is in a position other than α do not react, nor do peptides in which the free COOH and NH_2 groups are spatially distant. On the other hand, glutathione, which has a free α-NH_2 group adjacent to COOH, yields 1 mol. CO_2 (*vide infra*). It should be noted that certain other cyclic ketones, e.g. isatin, also react in a manner similar to that of ninhydrin with amino-acids [Grassmann & van Arnim, 1934].

Previous applications of the ninhydrin reaction to the quantitative determination of amino-acids have been colorimetric or photometric procedures. These are subject to the criticism that not all amino-acids produce the same colour under the same conditions, that numerous substances other than amino-acids yield colour [Neuberg, 1914], and that various substances, e.g. ammonium salts, interfere greatly with the character and intensity of the colour developed [Polonovski & Moreno-Martin, 1935]. The literature on these methods has been summarized by Mitchell & Hamilton [1929].

Details of procedure

Reagents. NaOH *c.* 0·5*N*, prepared as described by Van Slyke *et al.* [1933]. Lactic acid, *c. N*. A sat. solution of KH_2PO_4, freed from dissolved CO_2 by shaking briefly under reduced pressure or by boiling. Ninhydrin (triketohydrindene hydrate). Glycerol.

Apparatus. The combustion tube and condenser described for procedure "A" in the determination of total C by Van Slyke *et al.* [1933] for use in conjunction with the Van Slyke-Neill manometric gas machine. An Ostwald-Van Slyke pipette calibrated to deliver 1·5 ml. Glass beads.

Procedure. The solution to be analysed is made slightly acid and is freed from dissolved CO_2 by shaking in a flask at reduced pressure or by boiling. A 1–5 ml. aliquot containing 0·05–2·0 mg. available carboxyl CO_2 is introduced into the clean dry combustion tube, followed by 1 or 2 ml. sat. KH_2PO_4 and about 30 mg. ninhydrin. A glass bead is employed to reduce bumping. The combustion tube is immediately connected with the condenser, the walls of the ground glass joint being smeared with glycerol as a sealing fluid. The condenser is then attached to the manometric gas pipette into which have been introduced 2 ml. of the 0·5*N* alkali as described by Van Slyke *et al.* [1933]. The gas chamber and combustion tube are then connected by turning the stopcock, the mercury levelling bulb resting on the lower support. The contents of the combustion tube are gently heated with a micro-burner. Before the onset of boiling the mercury in the gas chamber is alternately raised and lowered several times, care being exercised to prevent bumping of fluid up into the condenser. After 3 min. gentle boiling the flame is removed and the mercury level is alternately raised and lowered to draw the CO_2 over into the alkali in the gas pipette. This must be done with some care at first in order to avoid bumping, heating of the stopcock and condensation of large amounts of vapour in the gas chamber, with consequent alteration of the volume of fluid over which the CO_2 is ultimately measured.[1] The

[1] Increase of this volume by 0·1 or 0·2 ml. affects the accuracy of the determination only slightly; indeed, bumping of a few drops of the fluid from the combustion tube into the pipette will not invalidate the analysis if the reaction of the absorbing fluid is still alkaline. The error introduced is that entering into the gas factors which are calculated on the basis that the fluid volume over which the CO_2 is measured is 3·5 ml.

mercury level is thus altered 10–15 times allowing it to reach the lower shoulder of the pipette.

The contents of the combustion tube are again gently boiled for 1 min., and the absorption carried out as before. The unabsorbed gas is then ejected as described by Van Slyke *et al.* [1933, p. 641] and the absorption of CO_2 repeated at a greatly reduced pressure by altering the mercury level another 10 times. The unabsorbed gas is again ejected, and a final absorption is made by again altering the mercury level 10 times. Under these circumstances the pressure during the final manipulation is about 50 mm. when the mercury level is at the 50 ml. mark.

The stopcock connecting the condenser and gas chamber is closed, the condenser disconnected, the stopcock capillary sealed with mercury and the last portion of unabsorbed gas ejected. The CO_2 is then liberated with lactic acid and the pressure measured before and after absorption with 5*N* alkali as described by Van Slyke *et al.* [1933]. Measurements are made at the 0·5 ml. or 2·0 ml. mark depending on the amount of CO_2 present.

Blank analyses are performed similarly except that ninhydrin is omitted. One boiling period is sufficient in the blank determination if there is no reason to suspect the presence of substances decarboxylated by heat alone. Duplicate analyses usually agree within 1–2%, and with practice somewhat greater precision is obtained. The time required to complete a determination is 15–20 min. Between analyses the condenser is rinsed with distilled water and allowed to drain. The combustion tube is rinsed with water, alcohol and ether, and dried, and the gas chamber is washed with dilute lactic acid in the usual manner.

Calculation: $P_{CO_2}=(p_1-p_2)-C$

$P_{CO_2}\times f_{CO_2}=$ mg. CO_2 in the sample analysed.

The (p_1-p_2) reading in the blank analysis is the C correction. The value of this correction has averaged about 10 mm. when measured at the 2·0 ml. mark for the reagents used in this laboratory. The factors f_{CO_2} are those derived by Van Slyke & Sendroy [1927, p. 141].

In relating the CO_2 measured to the amino-acid concentration in mixtures it should be noted that the presence in unknown proportions of aspartic acid and cystine introduces an inaccuracy into the expression of the concentration in millimols, as these acids yield 2 mol. CO_2 (*vide infra*). Analogous difficulties are encountered when a similar calculation is made from the N_2 evolved with HNO_2. This must be borne in mind in making any comparisons between amino-acid N and amino-acid CO_2 in the same solution.

Results of analyses of solutions of pure amino-acids

The results of analyses of 24 common amino-acids are given in Table I. For reasons irrelevant to this communication these acids were dissolved in saturated solutions of picric acid. Similar results are obtained in aqueous solutions. The recoveries are satisfactory save in three instances, alanine, serine and tryptophan. The reasons for incomplete recoveries of these have not as yet been ascertained. In the analysis of a mixture of amino-acids, e.g. a protein hydrolysate, imperfect behaviour of these three acids would introduce ordinarily only a very small error into the determination of the total amino acid content.

Cystine and aspartic acid each yield 2 mol. CO_2 whereas only one COOH group of glutamic and hydroxyglutamic acids reacts. Cystine, of course, presents two α-NH_2 groups adjacent to COOH. In the case of aspartic acid, the malonyl

Table I. *Analyses of picric acid solutions containing known amounts of pure amino-acids*

Compound	Quantity analysed mg.	Recovery calculated from CO_2 evolved %
Glycine	0·61	98·2
dl-Valine	0·89	98·8
l-Leucine	1·34	99·9
dl-*iso*-Leucine	0·93	101·0
dl-Cysteine	0·88	100·2
dl-Methionine	0·80	102·7
dl-α-Amino-isobutyric	0·62	99·7
dl-Phenylalanine	0·56	100·3
dl-α-Amino-phenylacetic	0·72	97·2
l-Tyrosine	0·48	98·0
l-Dihydroxy-phenylalanine	1·34	99·5
dl-Lysine	1·84	101·2
dl-Arginine	7·15	98·0
Asparagine	1·33	99·6
l-Histidine	1·07	98·3
l-Proline	0·59	102·6
l-Hydroxy-proline	0·66	101·7
dl-Glutamic acid	0·87	102·5
i-β-Hydroxyglutamic acid	2·84	103·7
l-Aspartic acid (2 mol. CO_2)	0·45	100·6
l-Cystine (2 mol. CO_2)	0·36	99·9
dl-Alanine	—	75–90
dl-Serine	—	87–97
l-Tryptophan	—	88–96

aldehyde resulting from oxidation by ninhydrin presumably immediately decarboxylates as a result of boiling to yield the second mol. CO_2. The second COOH of glutamic and hydroxyglutamic acids is not entirely insensitive, and gradually gives up CO_2 on prolonged boiling. With the boiling periods specified, recoveries are constantly obtained within 4% of theoretical for one COOH. The second COOH of hydroxyglutamic acid appears to be somewhat more reactive than that of glutamic acid.

This behaviour of aspartic and glutamic acids affords a rapid means of determining the quantities of each when they are separated as the mixed silver salts in the partition of protein hydrolysates. If both the NH_2-N (HNO_2 reaction) and the carboxyl CO_2 are estimated, the mol. CO_2 in excess of the mol. N_2 represent the aspartic acid.

Behaviour of ninhydrin with peptides and amino-acids other than α-amino-acids

Table II gives the results of analyses of a series of peptides. Preliminary analyses with 6 min. boiling indicated that a small amount of CO_2 equivalent to 1–4% of one COOH group was evolved. With the shorter boiling periods, totalling 4 min., much less is obtained. It seems probable that there is some hydrolysis of the peptide linkage with prolonged heating, although a very slow reaction with the ninhydrin is not excluded. Glutathione, which has an adjacent α-NH_2 and COOH group, yields 1 mol. CO_2 as anticipated. Also included in this table are results on two *N*-methylamino-acids, a β-NH_2 and ε-NH_2 acid. *N*-methyltyrosine was entirely unreactive; however, sarcosine evolved variable amounts (10–30%) of CO_2. The conditions employed by Van Slyke & Dillon [1938] apparently permit complete decarboxylation of the latter acid. The β- and ε-acids did not react.

Table II. *Analyses of solutions of peptides and amino-acids other than α-amino-acids*

Compound	Quantity analysed mg.	Recovery calculated from CO_2 evolved %
Glycyl-glycine	3·69	<3·0
dl-Leucyl-glycine	4·98	0
Glycyl-*l*-tyrosine	2·92	0
dl-Leucyl-glycyl-glycine	4·14	<1·4
Glycyl-*l*-tryptophan	2·94	0
dl-Alanyl-glycine	6·26	<2·0
Leucyl-*l*-tyrosine	3·32	<2·0
Glycyl-*dl*-leucine	3·96	<2·0
Glycyl-glycyl-glycine	4·18	<2·0
Glutathione (1 mol. CO_2)	6·53	98·4
dl-β-Amino-β-phenylpropionic	2·20	0
ε-Amino-*n*-hexanoic acid	3·23	0
N-Methyltyrosine	1·63	0
Methylglycine (sarcosine)	4·15	10–30

Development of intense colour is not a criterion of decarboxylation, and may occur without any CO_2 evolution. The colour reactions of ninhydrin with amino-acids and related substances under various conditions are discussed elsewhere [Neuberg, 1914; Grassmann & van Arnim, 1934; Mason, to be published].

Possible interfering substances

A number of common carboxylic acids, keto-derivatives of amino-acids and other compounds, were tested in order to ascertain whether or not they evolved CO_2 or interfered with the determination in any way. Most of these were entirely unreactive (α-ketoglutaric, mesoxalic, lactic, citric, fumaric, succinic, oxalic, acetic, butyric and benzoic acids) although several (pyruvic, oxaloacetic and acetoacetic acids) were decarboxylated by heating only, as evidenced by the value of the blank analysis. No additional CO_2 was evolved with ninhydrin with any of these acids; a trace (<1·0 %) of additional CO_2 obtained from pyruvic acid is probably due to some variation in amount of decomposition during the blank determination. However, in the case of glucosamine some of the aldehyde may be oxidized by ninhydrin and the resulting α-amino-acid behave in the usual way.

These data indicate that the measurement of carboxyl CO_2 liberated by ninhydrin should afford a highly specific means of estimating amino-acids in biological materials. The application of the method to analysis of blood filtrates, etc., is being studied further, and will be discussed in a subsequent report.

Summary

1. Amino-acids heated with ninhydrin in aqueous solution are decarboxylated.

2. A technique for measuring manometrically the CO_2 evolved in the Van Slyke-Neill gas machine has been described.

3. Of 24 common amino-acids tested, all yielded 1 mol. CO_2 save aspartic and cystine which yielded 2, and alanine, serine and tryptophan, which evolved slightly less than 1.

4. Peptides do not react unless they have free adjacent α-NH_2 and COOH groups.

5. Various common organic acids other than amino-acids were found not to yield CO_2 upon treatment with ninhydrin.

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XCIX. THE ACTIVATION OF FEMALE SEX HORMONES

II. α-OESTRADIOL AND ITS DI-ESTERS

By K. MIESCHER, C. SCHOLZ and E. TSCHOPP

From the "Ciba" Research Laboratories

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In the preceding paper of this series [1938] it was shown how the effect of oestrone can be systematically increased by suitable esterification. In these investigations special attention was directed to the testing of larger doses and the control of the duration of their effect. We now report on the results obtained in a similar way with α-oestradiol and its di-esters[1]. The effect of the 3- and 17-monoesters of oestradiol will be described in a later paper. It is possible to differentiate between symmetric and asymmetric di-esters of oestradiol according to whether the 2 acid radicals in the 3- and 17-positions are the same or of a different kind. The di-esters used for the investigations as well as some of their characteristics are listed in Table I.

Table I

α-Oestradiol di-esters	M.P.* or B.P.† °C.	Oestrus test (rat): Threshold value S_r oestradiol $=0{\cdot}4\,\gamma$	Oestrus test (rat): Threshold value S_o based on oestradiol $=0{\cdot}1\,\gamma$
Diacetate	127–128	0·5	0·1
3-Acetate-17-propionate	112·7–113	0·6	0·1
Dipropionate	104–105	0·75	0·2
Di-*n*-butyrate	64–65	3·5	0·9
Di-*iso*butyrate	100·5–101·5	6·0	1·5
Di-*n*-valerate	220–230 0·05 mm.	7·0	1·8
Di-*n*-hexanoate	220–240 0·0005 mm.	8·0	2·0
Di-*n*-octanoate	240 0·0005 mm.	10·0	2·5
Di-*n*-decanoate	260–265 0·0001 mm.	25·0	6·2
Dipalmitate	63–65	27·0	6·8
3-Benzoate-17-acetate	172–173	4·0	1·0
3-Benzoate-17-propionate	167–167·5	4·0	1·0
3-Benzoate-17-*n*-butyrate	128·5–129	9·0	2·2
3-Benzoate-17-*n*-valerate	133–133·5	17·0	4·2
17-Benzoate-3-acetate	161–161·5	8·0	2·0
17-Benzoate-3-propionate	165–166	28·0	7·0
17-Benzoate-3-*n*-butyrate	141·5–142	45·0	11·2
Dibenzoate	171·6–172	125·0	36·2

* The melting-points are taken with a short thermometer.
† Bath temperature.

The esters include representatives of the purely aliphatic, the mixed aliphatic-aromatic and the purely aromatic series. Only the diacetate, the 3-benzoate-17-acetate and the dibenzoate have been previously described. A report has been given elsewhere on the manufacture of eleven esters [Miescher & Scholz,

[1] As regards the preliminary communications in this field see the preceding paper p. 141, note 1.

1937]. Here we describe only those di-esters about which nothing has been published.

*Oestradiol-3:17-di-*n*-hexanoate.* A solution of 720 mg. of oestradiol in 4 ml. of pyridine was mixed with 1·06 g. *n*-hexanoyl chloride (3 mol.) and after 10–12 hr. it was heated to 70° for 30 min. Dilute acid was added and the oil which separated taken up in ether. The ethereal solution was washed, dried and filtered, and the solvent evaporated. The residue was an oil which was distilled twice at 0·0005 mm. (bath temperature 220–230°). All attempts to crystallize the distillate have failed. (Found: C, 76·33; H, 9·58 %. $C_{30}H_{44}O_4$ requires C, 76·86; H, 9·47 %.)

*Oestradiol-3:17-di-*n*-octanoate.* Oestradiol was dissolved in pyridine, mixed with 3 mol. *n*-octanoyl chloride and the reaction conducted as above. The oestradiol-3:17-di-*n*-octanoate obtained after the usual working up was oily and was purified by double distillation at 0·0005 mm. (bath temperature 240°). So far the yellow oil has not been crystallized. (Found: C, 77·88, 77·80; H, 10·26, 10·10 %. $C_{34}H_{52}O_4$ requires C, 77·99; H, 10·00 %.)

Oestradiol-3-acetate-17-propionate. 250 mg. oestradiol-17-propionate (obtained by partial saponification of oestradiol-3:17-dipropionate) were dissolved in 1·5 ml. of pyridine and mixed with 0·5 ml. acetic anhydride. After standing at room temperature for a short time it was heated to 100° for 30 min. and worked up in the same way. Oestradiol-3-acetate-17-propionate was recrystallized several times from methanol-water and formed fine crystals with constant M.P. 112·7–113°. (Found: C, 75·10; H, 8·21 %. $C_{23}H_{30}O_4$ requires C, 74·55; H, 8·17 %.)

Oestradiol-3-acetate-17-benzoate. The acetylation of oestradiol-17-benzoate (obtained by partial saponification of oestradiol-3-*n*-butyrate-17-benzoate) in pyridine with acetic anhydride was carried out in the usual way. Although it was recrystallized several times and had constant M.P. 161–161·5°, the substance was not analytically pure.

Test methods

These substances were investigated both as regards dosage and method of testing by the oestrus and uterus growth tests exactly as described in the previous paper. Young uncastrated rats were again used for the uterus growth test. Since, however, the actions of the majority of the esters last more than 30 days and since the animals, on account of the onset of sexual maturity, are then no longer suitable for this test, supplementary experiments were made using castrated rats. The results obtained will be completed at a later date.

The points given in the curves represent the average values obtained on at least 3 animals. 1350 animals were used for these experiments.

The assay in the oestrus test

Free oestradiol. On the basis of numerous experiments carried out according to our method, the threshold value of oestradiol dissolved in sesame oil was found to be 0·4 γ for the rat. The same figure was also obtained by Schoeller *et al.* [1935]. It is about half that for oestrone (0·7 γ). In experiments made by other authors values varying from 0·07 to 0·6 γ were found according to the technique used.

Fig. 1 shows that even with relatively large doses (50 γ) the effect of oestradiol is only of short duration. In this respect it is entirely similar to oestrone. Dissolved in sesame oil, palm-kernel oil or glycerol a total dose of 50 γ oestradiol ($2 \times 25\,\gamma$) causes 4 days' oestrus, but, dissolved in wool fat, 5 days.

Activators. Table II shows the influence of various activators on the duration of oestrus obtained with a total dose of 50γ oestradiol (in sesame oil) in the castrated rat.

Table II. *Effect of activators added to a dose of 2 × 25γ oestradiol*

Activators	Duration of oestrus in days
—	4
100 mg. Palmitic acid	5–6
200 ,, Palmitic acid	10 (7)*
200 ,, Ricinoleic acid	4–5
200 ,, Linoleic acid	4
200 ,, Cetyl alcohol	8
200 ,, Stearyl alcohol	24 (13)*

* The figures in brackets are for oestrone.

It will be seen that the activity of the true follicular hormone, oestradiol, is enhanced much more easily than that of the excretion product oestrone.

Di-esters

(*a*) *Aliphatic di-esters.* As will be seen from Table I and also from Fig. 1 the threshold values (S_t) determined on the rat for esters up to the dipropionate vary only slightly from that of free oestradiol, being $< 1\gamma$. With higher esters they rise quickly, reaching already with the di-*n*-decanoate 25γ which is almost the maximum, the dipalmitate having a value only 2γ more.

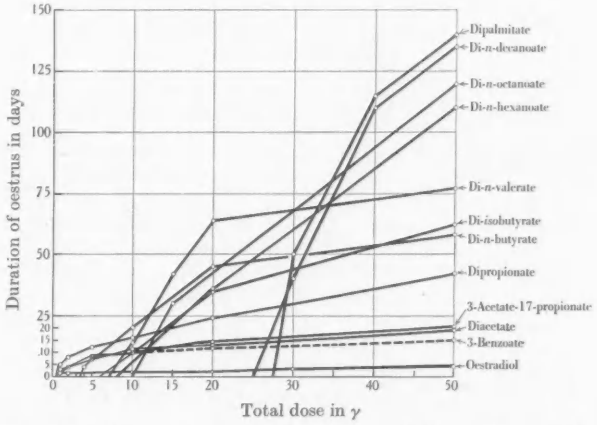

Fig. 1. The effect of oestradiol, oestradiol-3-benzoate and aliphatic di-esters of oestradiol on castrated rats in the oestrus test, using various doses.

It is clear from the curves of Fig. 1 that the longer the carbon chain of the acid used the greater the duration of oestrus obtained with the aliphatic di-esters. In general the duration of oestrus increases with the threshold value; with the lower di-esters the duration of effect increases more rapidly. Thus the duration of oestrus is 42 days with the dipropionate in a dose of 50γ whilst with the di-*n*-decanoate and the dipalmitate, the duration of effect amounts to almost 5 months.

In Fig. 2 the effects of the aliphatic oestradiol di-esters are compared with those of the corresponding oestrone esters as regards their threshold value (S_r) and duration of effect (T_{oe}). With the lowest esters of both series the threshold values are approximately the same; with the medium esters they are higher for oestradiol di-esters whilst for the highest esters the relationship is reversed. It must be observed that the number of carbon atoms of both acid radicals of the oestradiol di-esters is twice that of the corresponding oestrone esters. As regards duration of oestrus, the oestrone esters show a pronounced maximum; as we had assumed, the maximum occurs with the oestrone-*n*-heptanoate.[1] The oestradiol di-esters are far superior to the oestrone esters. With the former the steady increase in the duration of effect up to the di-decanoate is striking. A fall is only to be expected with still higher esters of oestradiol than those investigated.

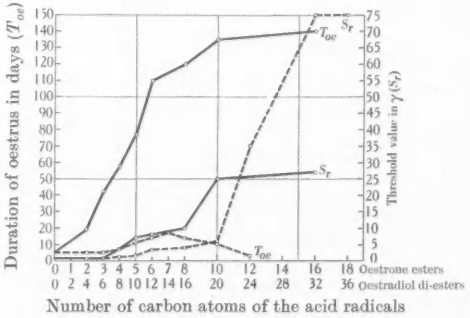
Duration of oestrus in days (T_{oe})
Threshold value in γ (S_r)
T_{oe}
S_r
S_r
T_{oe}
0 1 2 3 4 5 6 7 8 10 12 14 16 18 Oestrone esters
0 2 4 6 8 10 12 14 16 20 24 28 32 36 Oestradiol di-esters
Number of carbon atoms of the acid radicals

Fig. 2. Comparison of the aliphatic oestradiol di-esters [——] and oestrone esters [- - - -] as regards the threshold value (S_r) and duration of effect (T_{oe}) in the oestrus test.

Oestradiol dipropionate is of special interest as it exhibits with a low threshold value a relatively intense and prolonged effect which is dependent upon the dose. As a comparison it might be mentioned that the effect of oestradiol-3-benzoate, the only ester of oestradiol used clinically hitherto, does not quite attain that of the diacetate and is considerably below that of the dipropionate.

(*b*) *Aliphatic-aromatic esters.* This series is subdivisible into two according to whether the benzoyl radical is in the 3- or in the 17-position. Table I and Fig. 3 show clearly that the series of the 3-benzoyl esters has more favourable properties since the threshold value increases less quickly with the increasing length of the aliphatic acid radical than in the 17-benzoyl series. The 3-benzoyl series exhibits a more favourable action also as regards duration of effect. The 3-benzoate-17-*n*-butyrate and -17-*n*-valerate are of particular interest. As regards effect the former is similar to oestradiol di-*n*-octanoate but in contrast to this it crystallizes. The 3-benzoate-17-*n*-valerate is better than the dipalmitate which exhibits the most prolonged effect, in the aliphatic series, since it possesses the same duration of effect (50 γ : 140 days) with a smaller threshold value (17 γ against 27 γ).

[1] M.P. approx. 55°, B.P. 185–190° 0·0005 mm. (Found: C, 78·48; H, 8·96 %. $C_{25}H_{34}O_3$ requires C, 78·46; H, 9·15 %.) The threshold value in the oestrus test (rat) is $S_r = 2{\cdot}5$ γ, the duration of effect is 17 days (with a total dose of 50 γ). The output of activity in the uterus growth test is 202 cg.-days ($D_{max.} = 10{\cdot}2$ cg.; $t_{max.} = 4$th day; $T = 38$ days; $c = 16{\cdot}5$).

(c) *Aromatic di-esters.* Only the dibenzoate was tested. The threshold value is very high (125 γ). The duration of effect with a total dose of 200 γ, that is only 1·6 times the threshold dose, shows considerable variation; on the average it is about 30 days (5 animals).

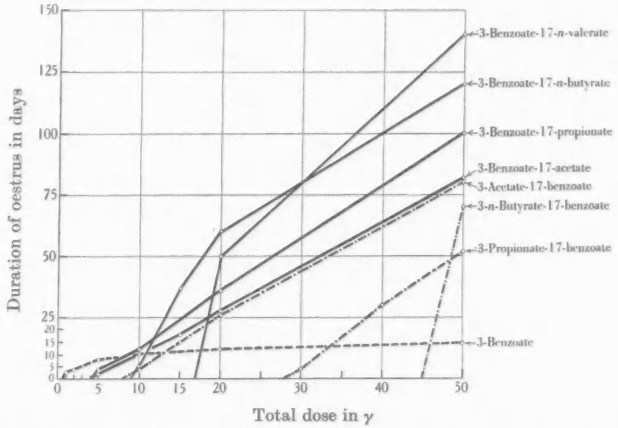

Fig. 3. The effect of mixed aliphatic-aromatic di-esters and oestradiol-3-benzoate on castrated rats in the oestrus test, using various doses.

Uterus growth test

As in the oestrus test oestradiol given by subcutaneous injection to young rats also exhibits a relatively transitory effect in the uterus growth test even in a dose of 50 γ (see Fig. 4). Nevertheless, the output of activity calculated according to the method given previously [1938] is almost double that for oestrone (20 cg.-days against 12 cg.-days). By the addition of stearyl alcohol a

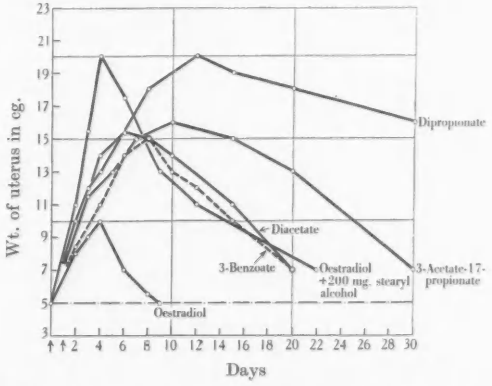

Fig. 4. The effect of oestradiol, with and without stearyl alcohol, and some lower aliphatic oestradiol di-esters and oestradiol-3-benzoate on the weight of the uterus of young rats after administration of a total dose of 50 γ of hormone given in equal parts on two consecutive days.

pronounced and rapid increase of effect is obtained (output of activity 151 cg.-days).

(*a*) *Aliphatic di-esters* (*young animals*). In this test the aliphatic oestradiol di-esters also show extraordinarily intense and prolonged effects (see Figs. 4 and 5). Even the diacetate is, as regards the time course of its effect, similar to the 3-benzoate. With a total dose of 50 γ a duration of effect of more than 30 days is attained with the 3-acetate-17-propionate and dipropionate as well as with all

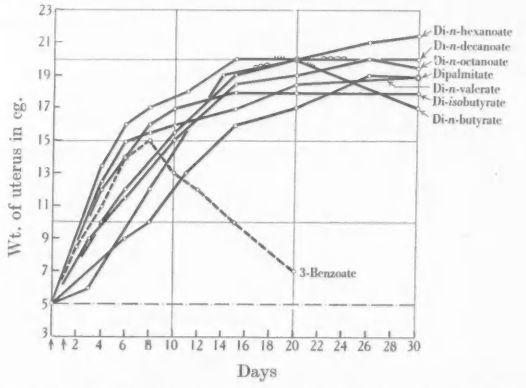

Fig. 5. The effects of some higher aliphatic di-esters of oestradiol and of oestradiol-3-benzoate on the weight of the uterus of young rats after administration of a total dose of 50 γ of hormone given in equal parts on two consecutive days.

di-esters with larger acid radicals. As has already been observed in the earlier tests with esters of oestrone, the total duration of effect on the uterus is more, in some cases considerably more, than that in the oestrus test. Higher esters such as the di-*n*-decanoate and dipalmitate doubtless exercise on the uterus an effect lasting about 6 months. The diacetate attains its maximum effect on the 6th day and the dipropionate on the 12th day; the maximum effect of the higher esters setting in still later. The increase of effect appears to be considerably delayed particularly with the dipalmitate.

(*b*) *Aliphatic-aromatic esters* (*young animals*). As in the oestrus test the aliphatic-aromatic esters show also in the uterus growth test a markedly protracted action (see Fig. 6). Even on the 30th day the total activity of all esters investigated is still maintained. The weights of the uteri attained vary between 190 and 220 mg. The increase of effect is as a rule quicker with the 3-benzoyl-esters than with the 17-benzoyl-esters. With the 3-benzoate-17-*n*-valerate it is delayed to the same extent as with the dipalmitate.

(*c*) *Aromatic di-esters.* While no total oestrus is obtained with 50 γ oestradiol dibenzoate, the same dose produces a slight increase in weight of the uterus, the maximal weight of the uterus being about 100 mg.

(*d*) *Tests with di-esters on castrated animals.* To supplement the tests on young animals the time course of effect of each of two aliphatic and mixed aliphatic-aromatic esters was examined on castrated rats also after 30 days (see Fig. 7). The values obtained follow on those observed on young animals. It will be seen that with the di-esters exhibiting prolonged effects, such as the di-*n*-decanoate, their maximal effects are maintained for a long time or, as with the

3-benzoate-17-*n*-butyrate, even continue to increase slowly. The latter ester produces, e.g. in a total dose of 50 γ, an average weight of the uterus of 230 mg. on the 50th day. These experiments will be extended.

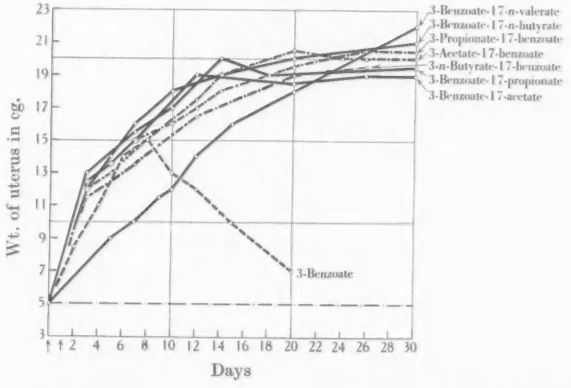

Fig. 6. The effect of mixed aliphatic-aromatic di-esters of oestradiol and of oestradiol-3-benzoate on the weight of the uterus of young rats after administration of a total dose of 50 γ hormone given in equal parts on two consecutive days.

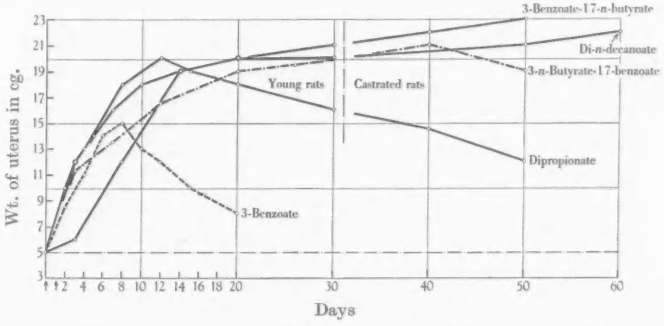

Fig. 7. The effects of aliphatic and mixed aliphatic-aromatic di-esters of oestradiol on the weight of the uterus of young uncastrated rats and castrated rats after administration of a total dose of 50 γ hormone given in equal parts on two consecutive days.

(*e*) *Systematic arrangement of the results of the experiments.* So far as the values obtained allow some of the compounds studied have been characterized systematically (see Table III) as was done with the esters of oestrone in the previous paper. Again the letters signify:

A, output of activity, $D_{\max.}$ the maximal increase in weight of the uterus, $t_{\max.}$ the day of the onset of the maximal effect, T the total duration of effect, D_m the average increase of weight of the uterus, G_o the weight of the uterus at the commencement of the experiment, $d_{\max.}$ the maximal increase in weight of the uterus in %, d_m the average % increase in weight of the uterus, and c the efficiency coefficient.

Table III

Substance	Absolute values: A cg.-days	$D_{max.}$ cg.	$t_{max.}$	T days	D_m cg.	Relative values (Based on $G_o=5$ cg.): $d_{max.}$ %	d_m %	Based on oestradiol =1: c
Oestradiol	20·3	5·0	4	9	2·3	100	46	1·0
Oestradiol + 200 mg. stearyl alcohol	150·6	15·0	4	27	5·6	300	112	7·4
Oestradiol diacetate	165·6	10·3	6	22	7·5	206	150	6·7
Oestradiol-3-acetate-17-propionate	278·8	11·0	10	33	8·5	220	169	13·7
Oestradiol dipropionate	567·2	15·0	12	60*	9·5	300	189	27·9
Oestradiol di-*n*-decanoate	1200·0*	—	—	80*	15·0	—	300	60·0
Oestradiol-3-benzoate	118·8	10·0	8	24	4·9	200	99	5·9

* Estimated.

Comparison with earlier experiments

Last year Parkes [1937] demonstrated the transitory effect of oestradiol in the plumage test on brown leghorns. According to his findings oestradiol diacetate was considerably better than oestrone acetate; oestradiol-3-benzoate-17-acetate proved to be the most effective, whilst oestradiol dibenzoate was effective only in very high doses. The high threshold value of the dibenzoate in the oestrus test (mouse) had also been observed by David *et al.* [1935]. Thus there is a satisfactory agreement between the findings of other authors and the results obtained on the rat by us with these compounds.

SUMMARY

1. Like free oestrone, free α-oestradiol exhibits only a transitory effect in the oestrus test on the rat even when given in high doses. Activators can considerably increase the duration of effect. In the uterus growth test oestradiol has a more intense effect than oestrone (output of activity 20 cg.-days against 12 cg.-days).

2. 10 aliphatic and 7 aliphatic-aromatic di-esters of α-oestradiol in addition to 1 aromatic di-ester were tested on the rat by subcutaneous injection (aromatic radical = benzoyl).

3. *Oestrus test.* In the aliphatic series the threshold value increases at first slowly up to the dipropionate and then more quickly up to the di-*n*-decanoate and dipalmitate. All di-esters exhibit very prolonged effects. The effect of the mixed aliphatic-aromatic di-esters is somewhat similar to that of the aliphatic di-esters with a similar number of carbon atoms. Nevertheless, the 3-benzoyl esters with an aliphatic acid radical in the 17-position are superior to their isomeric 17-benzoyl-di-esters.

4. *Uterus growth test.* In the uterus growth test using uncastrated young rats and also castrated rats all di-esters tested exhibit extremely intense and prolonged effects.

5. The oestradiol di-esters have on the whole much more intense and more prolonged effects than are attained even with the best oestrone esters as, e.g. oestrone-*n*-heptanoate.

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C. INTERACTION OF MALEIC ACID WITH THIOL COMPOUNDS

By EDWARD JAMES MORGAN and ERNST FRIEDMANN

From the Biochemical Laboratory, Cambridge

(*Received 5 February 1938*)

It was observed by one of us (E. J. M.) that the titratable SH of glutathione was greatly decreased by incubation with maleic acid in absence or in presence of oxygen at *p*H 7·4 and 37° (but not with fumaric acid). Thiolacetic acid and cysteine behaved similarly to glutathione. The results contained in this paper show that the disappearance of the SH-groups is due to the circumstance that each of the three substances studied yields an addition compound with maleic acid in the formation of which these groups are involved. The kinetics of such reactions show points of special interest.

Titration experiments

The following solutions were prepared: (1) a 0·2 *M* solution of the Na salt of the unsaturated acid (A); (2) a solution of the Na salt of the thiol compound such that 1 ml. was equivalent to 40 ml. 0·01 *N* iodine, i.e. 0·4 *M* with respect to SH (B). For experiment *x* ml. of A and *y* ml. of B were placed in each of a series of Thunberg tubes together with Sørensen's phosphate buffer, *p*H 7·4 (1 ml. for every 4 ml. final volume) and water to the required volume. The tubes were evacuated and incubated at 37°.

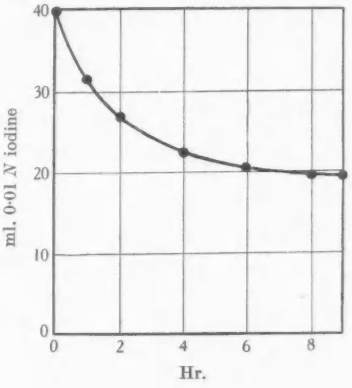


Fig. 1.

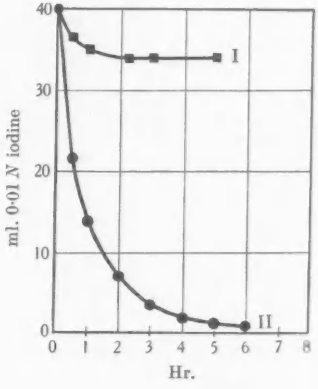


Fig. 2.

Fig. 1. Final concentration *M*/50 thiolacetic and *M*/50 maleic acid.

Fig. 2. Curve I, final concentration *M*/12·5 thiolacetic and *M*/50 maleic acid. Curve II, final concentration *M* 12·5 maleic and *M*/50 thiolacetic acid.

At successive intervals a tube was removed and its contents analysed iodimetrically for SH after acidification with trichloroacetic acid.

The interaction of maleic and thiolacetic acids is illustrated in Figs. 1 and 2 in which residual SH concentration in terms of ml. 0·01 *N* iodine is plotted against time. It will be seen that with equimolar concentrations of the reactants 50 % of the thiolacetic acid disappears (Fig. 1); with a molar ratio of thiolacetic to maleic acid of 4 : 1 on the other hand the disappearance of thiolacetic acid is only 15 % (Fig. 2, curve I) whilst with a ratio of 1 : 4 its removal is almost quantitative (Fig. 2, curve II).

Fig. 3 illustrates the comparative interactions of equimolar solutions of maleic and thiolacetic acids (Curve II), maleic acid and glutathione (Curve III) and maleic acid and cysteine (Curve V); it will be seen that thiolacetic acid and glutathione behave similarly whilst cysteine is more completely removed under these conditions. Curve IV shows that cyanide does not affect the reaction. No interaction was observed on substituting for maleic acid any of the following: fumaric acid (Fig. 3, curve I), citraconic, mesaconic, *cis*- and *trans*-cinnamic and α-phenyl-β-styrylmaleic [Friedmann & v. Heyningen, 1936] acids.

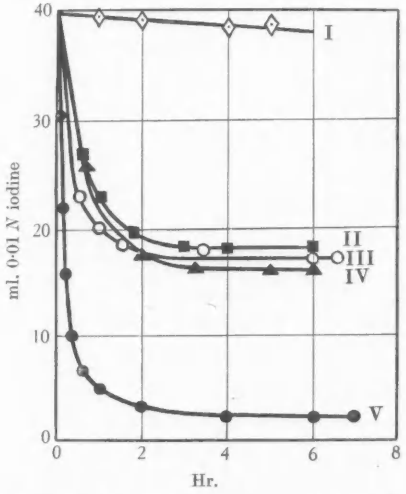


Fig. 3. Final concentrations. Curve I, *M*/12·5 thiolacetic and *M*/12·5 fumaric acids. Curve II, *M*/12·5 thiolacetic and *M*/12·5 maleic acids. Curve III, *M*/12·5 glutathione and *M*/12·5 maleic acid. Curve IV, *M*/12·5 thiolacetic acid, *M*/12·5 maleic acid and *M*/1000 KCN. Curve V, *M*/12·5 cysteine and *M*/12·5 maleic acid.

Polarimetric experiments

M/5 solutions were prepared of the Na salts of glutathione and of various unsaturated acids, the solutions containing 2 ml. of phosphate buffer, *p*H 7·4, in a total volume of 10 ml. For the experiments equal volumes of the solution of glutathione and that of one of the unsaturated acids were mixed and compared polarimetrically (Hg_{green}, 2 dm. tube, 21·5°) with controls consisting of glutathione solution mixed with an equal volume of a 1 : 5 dilution of Sørensen's phosphate buffer, *p*H 7·4.

Fig. 4, curve I illustrates the interaction of maleic acid and glutathione whilst curve II of this figure again demonstrates the inability of fumaric acid to replace maleic acid in the reaction; similar failure to react was observed with *cis*- and *trans*-cinnamic acids.

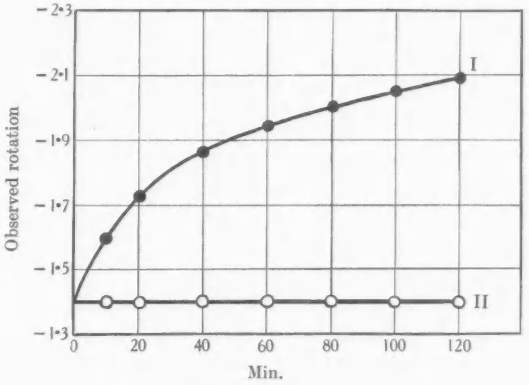


Fig. 4. Curve I, *M*/10 glutathione and *M*/10 maleic acid. Curve II, *M*/10 glutathione and *M*/10 fumaric acid. *t* 21·5°.

Kinetic experiments

In Table I are given the results of an attempt to study the kinetics of the interaction of maleic acid and glutathione in equimolar proportions. The solutions were prepared as described above and the experiments were carried out in open tubes, the reactants being brought to the bath temperature before mixing and the reaction being followed iodimetrically. The decrease of the reaction constants with time indicates a complex reaction, a supposition which is confirmed by examination of the reaction products.

Table I. *M/10 glutathione and M/10 maleic acid*

Time min.	Titratable SH 0·01 *N* I ml.	Decrease of titratable SH 0·01 *N* I ml.	$K' \quad \frac{1}{t}\log\frac{a}{a-x}$	$K'' \times 10^3 \quad \frac{x}{t\,(a-x)\,a}$
		A. Temp. 20°		
0	20·0	—	—	—
2	19·4	0·6	0·66	0·773
4	18·8	1·2	0·67	0·798
8	18·0	2·0	0·57	0·694
16	16·4	3·6	0·54	0·686
32	14·5	5·5	0·44	0·593
64	11·2	8·8	0·40	0·614
		B. Temp. 30°		
0	20·0	—	—	—
2	18·8	1·2	1·34	1·596
4	17·8	2·2	1·265	1·545
8	16·25	3·75	1·128	1·442
16	14·3	5·7	0·910	1·246
32	12·1	7·9	0·671	1·020
64	9·4	10·6	0·504	0·881

Products of the reaction

For separation of the reaction products use was made of the facts that unchanged thiol compound and the reaction product were precipitated by mercuric sulphate whilst the thiol compound alone was precipitated by cadmium acetate.

Maleic acid and thiolacetic acid.

Isolation and properties of reaction product. A solution of maleic acid (9·282 g.), neutralized with *N* NaOH to *p*H 7·4, and diluted to 400 ml. was mixed with one of thiolacetic acid (7·149 g.), similarly neutralized, in water (200 ml.), and 400 ml. of water in a Büchner flask. The flask was evacuated and incubated for 22 hr. at 37° after which 3·395 g. unchanged thiolactic acid (47·5 %) remained.

The solution was acidified with H_2SO_4 to Congo red and precipitated with $HgSO_4$, avoiding excess. The Hg-precipitate was collected and decomposed with H_2S as usual. After removing H_2S with a stream of hydrogen and centrifuging HgS, the supernatant fluid was made up to 990 ml. (iodine equivalent unchanged), and treated with cadmium acetate (10 %) avoiding excess; the filtrate from the cadmium acetate precipitate was reprecipitated with $HgSO_4$. After decomposing the precipitate with H_2S and removing H_2SO_4 from the filtrate quantitatively with $Ba(OH)_2$, the solution was concentrated under reduced pressure, transferred to a crystallizing dish and evaporated *in vacuo* over H_2SO_4. A white, crystalline, uniform product (8·4621 g., i.e. theoretical yield allowing for the thiolacetic acid recovered) remained, consisting of rosettes of needles. It was recrystallized from a little water and analysed after drying *in vacuo* over P_2O_5 at 40°. (Found: 34·7; H, 4·0; S, 15·1 %. $C_6H_8O_6S$ requires C, 34·6; H, 3·9; S, 15·4 %.) M.P. 140·5° (uncorr.) after softening at 135°.

The substance contains no water of crystallization. It is readily soluble in cold water, methanol, ethanol and acetone, less soluble in cold ether; ethyl acetate and glacial acetic acid dissolve it sparingly in the cold, better on warming. Chloroform, benzene, toluene, xylene and light petroleum (B.P. 50–60°) scarcely dissolve it, even on heating.

Preparation, analysis and reactions indicate that the new substance is an addition compound of thiolacetic and maleic acids, of the formula

$$HOOC.CH_2.CH(COOH).S.CH_2.COOH.$$

In accordance with this structure it titrates as a tricarboxylic acid: 0·0416 g., required 6·0 ml. 0·1 *N* NaOH to phenolphthalein, giving equiv. 208·0: $C_6H_8O_6S$ titrating as a tribasic acid requires 208·1.

Further evidence for the assumed constitution is provided by alkaline hydrolysis of the substance. Molecular proportions of the addition compound, *N* NaOH and benzyl chloride were heated at 70°, transferred to a thermos flask and shaken for 8 hr. The acidified solution deposited an oily precipitate which was discarded. The filtrate on evaporation at room temperature gave a crystalline mixture which was dissolved in alkali, extracted with ether in order to remove traces of dibenzyldisulphide, acidified and again extracted with ether. This ethereal extract gave, after removal of the solvent, needles and plates which could be separated mechanically under a lens or better by extraction with benzene which dissolved the plates. The undissolved needles were recrystallized from water and proved to be fumaric acid. M.P. 280° (uncorr.). (Found: C, 41·6; H, 3·6 %. $C_4H_4O_4$ requires C, 41·4; H, 3·5 %. 0·0116 g. neutralized 2 ml. 0·1 *N* NaOH to phenolphthalein; $C_4H_4O_4$ (dibasic) requires 2 ml.).

The evaporated benzene solution gave a crystalline substance which, after purification by sublimation *in vacuo*, and recrystallization from dilute alcohol, softened at 59° and melted at 62°. Gabriel [1879] gives the M.P. of benzylthiolacetic acid as 58–59°; Andreasch [1897] gives 60°. (Found: C, 59·2; H, 5·5; S, 17·4%. $C_9H_{10}O_2S$ requires C, 59·3; H, 5·5; S, 17·6%. 0·0182 g. neutralized 1·0 ml. 0·1 *N* NaOH to phenolphthalein; $C_9H_{10}O_2S$ requires 1·0 ml.)

Isolation of thiolacetic acid. The reaction solution from a similar experiment with maleic and thiolacetic acids (residual thiolacetic acid by titration = 3·22 g.) was completely precipitated with cadmium acetate; the precipitate was collected and decomposed as usual and the filtrate evaporated in a vacuum desiccator over H_2SO_4; the residue was taken up in ether and the ether solution dried and evaporated, yielding 2·6 g. (81%) of thiolacetic acid, B.P. 108–109°/14 mm. The loss is due to partial volatilization in the desiccator.

Isolation of fumaric acid. The filtrate and washings from the $HgSO_4$ precipitation of the reaction solution obtained as above were freed from mercury with H_2S and concentrated under reduced pressure to about 100 ml.; on keeping in a refrigerator 3·965 g. of colourless needles were obtained which gave three successive crops from hot water (2·615, 0·769, 0·038 g.) all having M.P. 280°, not depressed by admixture with fumaric acid. (Found: C, 41·6; H, 3·7%. $C_4H_4O_4$ requires C, 41·4; H, 3·4%.) Since 4·25 g. maleic acid correspond to the 3·22 g. thiolacetic acid found not to have reacted, the yield of fumaric acid is 93·3%.

Maleic acid and cysteine

2·428 g. cysteine were dissolved in 200 ml. *M*/2·5 maleic acid and neutralized with *N* NaOH to *p*H 7·4, the final concentrations thus being *M*/2·5 maleic acid and *M*/10 cysteine. After evacuation, the solution was kept for 8 hr. at 37° and then at room temperature overnight. In the morning only a faint nitroprusside reaction remained. The solution was precipitated with $HgSO_4$, and the precipitate collected and decomposed as usual; the filtrate was evaporated *in vacuo*, yielding 4·728 g. (calculated 4·749 g.) of an amorphous product. For analysis the substance was dissolved in water, the solution frozen and dried by Pirie's method [1931] which gave a friable product. It was finally dried at 40° over P_2O_5. (Found: C, 35·8; H, 4·95; N, 6·0; S, 14·7%. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9; S, 13·5%. 0·0474 g. neutralized 4·0 ml. 0·1 *N* NaOH to phenolphthalein, giving equiv. wt. 237; $C_7H_{11}O_6NS$ titrating as a dibasic acid has equiv. wt. 237·05.)

The substance softens at 102° and melts at 134–135° (decomp.). By analogy with the product of reaction between maleic and thiolacetic acids the constitution of the new compound can be assumed to be:

$$HOOC.CH_2.CH(COOH).S.CH_2.CH(NH)_2.COOH.$$

Maleic acid and glutathione

50 ml. of *M*/5 maleic acid and 50 ml. of approximately *M*/5 glutathione, both neutralized with *N* NaOH to *p*H 7·4, were mixed and kept under vacuum at 37° for 8 hr.; 1·166 g. glutathione remained (determined iodimetrically).

The $HgSO_4$ precipitate, worked up as before, gave a hygroscopic substance (2·402 g.; calculated 2·410 g.). It seems to contain water of crystallization which can be removed only with difficulty. (Found: N, 8·8; S, 6·5%. $C_{14}H_{21}O_{10}N_3S, 3H_2O$ requires N, 8·8; S, 6·5%. After drying *in vacuo* over H_2SO_4 found: C, 37·2; H, 5·6%. $C_{14}H_{21}O_{10}N_3S, 1\frac{1}{2}H_2O$ requires C, 37·3; H, 5·3%. Dried 14 days *in vacuo* over P_2O_5, 0·0423 g. neutralized 3·1 ml. of 0·1 *N* NaOH to phenolphthalein,

corresponding to equiv. wt. 409·1, the theoretical value for $C_{14}H_{21}O_{10}N_3S$, titrating as a tribasic acid being 423·2.)

The substance dried over P_2O_5 softens at 122° and melts at 151–152° (decomp.). The analysis indicates a composition analogous to the addition compounds described above, and the compound may be formulated:

$$HOOC.CH_2.CH(COOH).S.G.$$

The cadmium precipitate, decomposed as before, gave glutathione in nearly theoretical yield (1·163 g.). It was identified by its M.P. 190° (decomp.), its iodine value and by analysis. (Found: N, 13·4; S, 10·3 %. $C_{10}H_{17}N_3SO_6$ requires N, 13·4; S, 10·5 %.)

The $HgSO_4$ filtrate gave 0·474 g. fumaric acid (calc. 0·499 g.).

Rearrangement of maleic acid to fumaric acid during the reaction

Fumaric acid has been shown above to be a reaction product of maleic and thiolacetic acids and of maleic acid and glutathione. The truth of this interpretation had to be checked since Skraup [1891] has shown that the metal salts of maleic acid when treated with H_2S give fumaric as well as maleic acids. We therefore investigated the degree of rearrangement due to the manipulations during the isolation of maleic acid in our experiments.

3 g. maleic acid, 0·97 g. Na_2SO_4, 20 ml. 10 % cadmium acetate, 20 ml. $HgSO_4$, 3 ml. glacial acetic acid and 30 ml. conc. H_2SO_4 in 1500 ml. distilled water were kept at room temperature for 4 days. The solution was treated with H_2S and worked up for unsaturated acids as above. The fumaric acid isolated amounted to 0·1098, i.e. only 3·6 % of the maleic acid employed.

Conclusive evidence that fumaric acid is a true product of reaction formed during the interaction of maleic acid with certain thiol-compounds is provided by its direct isolation as follows. The reaction solution prepared as usual from glutathione (0·5986 g.) and maleic acid (0·232 g.) had lost 0·3530 g. glutathione, equivalent to 0·1309 g. maleic acid; thus 0·89 g. maleic or fumaric acid was contained in the remaining 22 ml. of solution. The latter was acidified with 5·3 ml. *N* H_2SO_4 and extracted with ether for 8 hr. The ether extract gave after evaporation from a water bath not above 40° a crystalline residue (0·76 g.) which, after recrystallization from hot water proved to be pure fumaric acid, M.P. 280°. (Yield 92·8 %.)

In a control experiment in which glutathione solution was replaced by water the ether extract gave 0·207 g. maleic acid (89·2 %).

For the interpretation of the results it seemed necessary to investigate whether the observed conversion of *cis*-acid into *trans*-acid was connected with addition of the thiol groups to the double bond or whether the mere presence of a thiol compound suffices to induce the rearrangement. Since *cis*-cinnamic acid has been shown not to react with glutathione the incubation of *cis*-cinnamic acid with glutathione was examined.

cis-Cinnamic acid (M.P. 58°, 0·296 g. in 10 ml.), glutathione (0·614 g. in 5 ml.), both neutralized to *p*H 7·4, and 5 ml. of water, were incubated together *in vacuo* at 37° for 6 hr.; the solution was then acidified with 4 ml. *N* H_2SO_4 and extracted continuously with ether for 8 hr. The ethereal extract, dried with Na_2SO_4, gave a residue which was treated with aniline [cf. Liebermann, 1890, 1891]. The aniline salt so obtained was washed with light petroleum and dried (0·37 g.). It melted at 83–84°. The aniline salt was suspended in water, decomposed with *N* HCl and the free acid extracted with ether. The ethereal extract, washed with

water and dried with Na_2SO_4 gave, after removal of the solvent *in vacuo*, a residue which was dissolved in light petroleum, B.P. under 40°. By slow concentration three fractions were obtained (0·130; 0·062; 0·021 g.) which were identical. They had M.P. 58°, not depressed in mixture with *cis*-cinnamic acid. Yield of pure *cis*-cinnamic acid 72%.

It is clear therefore that a thiol compound which cannot be added to the double bond of an unsaturated *cis*-acid is unable to induce the rearrangement from the *cis*- to the *trans*-configuration under the experimental conditions described.

Yield of the end products of the reaction between maleic acid and thiol compounds

Over the concentration range so far studied (*M*/50–*M*/10) the yield of the addition product of maleic and thiolacetic acids increases with increasing concentrations. In order to determine the maximum yield of the addition product we therefore investigated the interaction at *M*/2 concentration which is the highest compatible with the solubility of sodium maleate. *M* maleic acid (Kahlbaum) (40 ml. with 20 ml. 4*N* NaOH, 8 ml. phosphate buffer *p*H 7·4 and 12 ml. water) and *M* thiolacetic acid (B.D.H.) (40 ml. with 10 ml. 4*N* NaOH 8 ml. buffer and 22 ml. water) were warmed to 37° and mixed in vacuum tubes which were at once evacuated, each tube containing 4 ml. of the mixed solution. The tubes were incubated at 37° and removed at intervals for titration. The results are given in Table II.

Table II. M/2 *maleic and* M/2 *thiolacetic acids at 37°*

Time	0·1*N* iodine ml.
Initial	20
15 min.	9·0
30 min.	7·8
45 min.	7·2
1 hr.	6·9
2 hr.	6·2
3 hr.	5·8
4 hr.	5·7

The reaction is complete after 3–4 hr., 72·2% of the addition compound having been formed. The experiment shows therefore that by increasing the concentrations of the reactants from *M*/10 to *M*/2 the yield of the addition compound is still further increased.

The yield of fumaric acid, formed during the reaction, has so far been determined only for *M*/10 concentrations of each reactant. For the interpretation of the reactions involved it seemed desirable to study the rearrangement (*a*) at *M*/50 for both reactants and (*b*) at a concentration of maleic acid compensating the loss of maleic acid by its rearrangement to fumaric acid. The latter conditions are fulfilled by the interaction of *M*/12·5 maleic acid with *M*/50 thiolacetic acid.

(*a*) 2·320 g. maleic acid and 1·842 g. thiolacetic acid were neutralized and made up to 100 ml.; 5 ml. required 10 ml. of 0·01*N* iodine. The mixture was incubated for 15 hr. at 37°; 5 ml. of the incubated solution required 4·3 ml. 0·01*N* iodine, equivalent to 43% of the original thiolacetic acid. The $HgSO_4$ precipitate of this solution, worked up as above, gave 0·8215 g. fumaric acid. Allowing for the solution used for titration this corresponds to formation of 35·8% fumaric acid as against a calculated amount of 42·8%.

(*b*) 9·282 g. maleic acid and 1·842 g. thiolacetic acid were neutralized and made up to 100 ml. The mixture was incubated for 17 hr. at 37°; 20 ml. of the incubated solution required no iodine. Thus the thiolacetic acid was completely removed during the reaction. The $HgSO_4$ precipitate gave 1·8176 g. or 19·6 % fumaric acid, and 5·0973 g. or 54·9 % maleic acid both corrected for samples removed. As 2·3205 g. or 25 % maleic acid were used for the formation of the addition compound 9·2314 g. or 99·5 % of the maleic acid is accounted for.

The results showing the yields of the end products from the interaction of maleic acid with thiolacetic acid under different molar concentrations of the reactants are summarized in Table III.

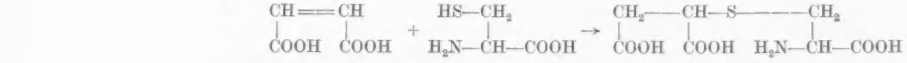
Table III

	Concentrations of the reactants			End products				
				Thiolacetic acid		Maleic acid		
Exp. no.	Maleic acid	Thiol-acetic acid	Time of incu-bation hr.	In addition compound %	Free %	In addition compound %	Free %	Fumaric acid formed from maleic acid %
1	*M*/2	*M*/2	4	72·2	27·8	72·2	—	—
2	*M*/10	*M*/10	19	65	35	65	0	34·2
3	*M*/12·5	*M*/12·5	4	50	47	53	—	—
4	*M*/12·5	*M*/12·5	22	55	45	55	—	—
5	*M*/50	*M*/50	24	54·5	45·5	54·5	—	—
6	*M*/50	*M*/50	15	57	43	57	0	Found 35·8 Calc. 42·8
7	*M*/12·5	*M*/50	5	98·8	1·2	24·7	54	—
8	*M*/12·5	*M*/50	17	100	0	25	—	19·6
9	*M*/50	*M*/12·5	5	15	85	60	—	—

Discussion

The results of the interaction of maleic acid with thiol compounds (at *M*/12·5 concentration) vary with the individual thiol compounds employed. The addition compound from cysteine is obtained in a yield of 93·5 %, whilst that from thiolacetic acid and glutathione is formed in yields of 55 and 56·5 % respectively (Fig. 3). Furthermore during the interaction of thiolacetic acid and glutathione with maleic acid a considerable amount of maleic acid is rearranged to fumaric acid.

The simpler case is obviously the reaction between cysteine and maleic acid, where in view of the large yield of the addition compound (93·5 %), the interaction can be formulated as a simple addition reaction of cysteine and maleic acid:



The greater reactivity of cysteine as compared with those of thiolacetic acid and glutathione is analogous with the similar behaviour of these substances towards iodoacetic acid [Smythe, 1936].

The interpretation of the interactions of maleic acid with thiolacetic acid and with glutathione is more complicated. Table III shows that within the range of concentrations *M*/50–*M*/12·5 the reactions proceed on very similar lines, 54–57 % of the theoretical yield of the addition compound being formed and 43–47 % thiolacetic acid remaining uncombined after incubation. At higher concentrations the yield of the addition compound increases. This is shown in Fig. 5 for the concentrations *M*/50, *M*/12·5 and *M*/2.

Thus at higher concentrations the reaction resembles more and more the simple addition demonstrated in the case of cysteine with maleic acid.

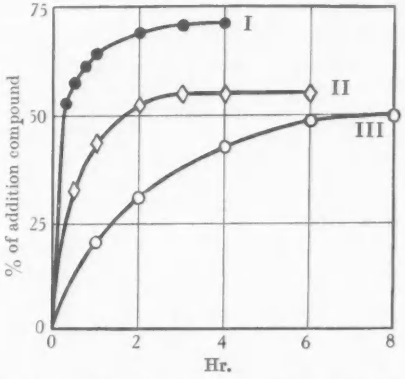


Fig. 5. Curve I, $M/2$ thiolacetic and $M/2$ maleic acids. Curve II, $M/12{\cdot}5$ thiolacetic and $M/12{\cdot}5$ maleic acids. Curve III, $M/50$ thiolacetic and $M/50$ maleic acids.

At concentrations between $M/12{\cdot}5$ and $M/50$ the interpretation has to account for the rearrangement of maleic acid to fumaric acid. The simplest formulation would be:

2 maleic + 1 thiolacetic = 1 addition product + 1 fumaric.

which implies that, for non-equimolecular proportions of reactants, the ratio of fumaric acid, formed by rearrangement, to maleic acid in the addition compound ought to be 1 : 1 and that for the experiment with $M/12{\cdot}5$ maleic acid and $M/50$ thiolacetic acid the ratio of fumaric acid: combined maleic acid: free maleic acid should be 1 : 1 : 2. Actually, as will be seen from Table III, the amounts of fumaric acid found are considerably lower than required by these ratios.

This observation can be explained by the assumption that the simple addition reaction which is predominant at higher concentrations takes place to some extent also at lower concentrations. Two main reactions thus proceed simultaneously: (1) simple addition and (2) addition plus rearrangement. The question whether low concentrations of the reactants favour the second reaction as suggested by Exp. 6, Table III, will be investigated later.

The reaction described in this paper is a new example of the well established fact that reagents which are able to rearrange an ethylenic linkage can form addition compounds at the double bond. The reaction described can be applied to the investigation of biochemical processes in which SH-compounds play an important role. Work in this direction has already been done, and is continuing. In particular experiments on the succinodehydrogenase of muscles, on papain, on glyoxylase and on the non-phosphorylating breakdown of glucose which is activated by glutathione, have been carried out. The results obtained will shortly be described.

SUMMARY

Maleic acid reacts with thiol compounds with the formation of addition compounds. In the case of thiolacetic acid and glutathione, maleic acid is rearranged simultaneously to fumaric acid.

The addition compounds of maleic acid with thiolacetic acid, cysteine and glutathione have been isolated.

The mechanism of the reactions involved has been examined and discussed.

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CI. DETERMINATION OF THE KETOGENIC ACTIVITY OF EXTRACTS OF ENDOCRINE ORGANS

By CHARLES HORACE GRAY[1]

From the Graham Research Laboratories, University College Hospital Medical School, London

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Numerous workers have reported the ketogenic effects of crude extracts of the anterior hypophysis. Burn & Ling [1933] found that an alkaline extract of fresh glands after neutralization with acetic acid increased total ketonuria in seven rats out of seventeen fed on a high fat diet. All seven were females, and no rise was recorded in male rats. This work was confirmed by Black *et al.* [1934], who showed that ketogenic responses to extracts of the anterior pituitary could also be obtained in fasting rats.

Meanwhile, Funk [1933] had isolated a material by benzoic acid adsorption from pregnancy or normal urine which caused an increase in ketonuria of rats; it is not clear from his paper whether these were fasting or on a fat diet. Butts *et al.* [1934] have shown that extracts prepared according to Burn and Ling greatly exaggerate the ketonuria of fasting rats and that of rats fed with acetoacetic acid. As marked responses were obtained with male as with female rats, and with castrated animals as with normal ones. Best & Campbell [1936] using a neutralized alkaline pituitary extract precipitated with alcohol, again confirmed the ketogenic effect on fasting female rats, and noted in addition a very great increase of liver fat.

Parallel with this work, there have been numerous attempts to determine the activity of similar extracts by their power to increase ketonaemia in rats and rabbits. Anselmino & Hoffmann [1931] showed that simple extracts of dried anterior hypophysis produced a rise in the blood ketones of non-fasting rats. The active constituent was water-soluble, but insoluble in ether, chloroform and in high concentrations of alcohol; it was ultrafiltrable and thermolabile. Magistris [1932] confirmed this work using rabbits, and Best & Campbell [1936] obtained similar results using a neutralized acid extract of fresh glands. The degree of ketonaemia showed large variations which bore no constant relation to the dose administered. The extract used also increased liver fat in female rats, but less markedly than did alkaline extracts of the same powder. Unfortunately these workers do not report the ketogenic activity of their acid extract in rats. They state also that "the rise in blood ketones (of the rabbit) was also noted after thyroidectomy up to the eighteenth day". After 18 days the response was negative, but returned after the administration of thyroid by mouth.

Black [1935] and Bennett [1937] have shown that crude extracts lose their ketogenic activity when administered daily over a period of several months to rats fed on a normal diet. The animals were fasted for 2 or 3 days at intervals in order to determine the ketogenic efficacy of the extracts. Anselmino & Hoffmann [1936, 1] have confirmed their previous work and have shown further

[1] Graham Scholar, University of London.

that extracts of sheep anterior pituitaries are more strongly ketogenic than those from cattle and the latter more so than those from the pig.

Thus, there appears little reason to doubt that there is present in the anterior lobe of the hypophysis a factor which stimulates ketosis. The well-known association between ketosis and fat deposition in the liver suggests that the ketogenic factor might be identical with that considered to increase the liver fat of fasting female rats.

More recently extracts of other organs have been found to possess ketogenic activity, e.g. ovarian extracts [Anselmino & Hoffmann, 1936, 2] and massive doses of adrenal cortical extracts [MacKay & Barnes, 1936]. Thus the point is raised as to whether ketogenic activity is shared by a number of endocrine organs, or whether there is one particular organ directly controlling ketogenesis, this organ being in its turn under the control of some other organ. The work of Long & Lukens [1936] stresses the importance of the role played by the adrenal cortex in ketogenesis.

In view of the inconsistent responses obtained by previous workers it becomes important to determine a method of estimating ketogenic activity giving the most consistent results in a simple manner.

Methods. I. Chemical

Preparation of anterior pituitary extract

Aqueous extract. 1 g. of acetone-dried ox anterior lobes was triturated with a little water in a mortar and the volume gradually made up to 20–25 ml. The supsension was then stirred mechanically for 12–24 hr., centrifuged and the supernatant injected, 10 ml. into a rabbit or 3 ml. into a rat.

Saline extract. A simple saline extract of fresh ox anterior lobes prepared by Dr F. G. Young of The National Institute for Medical Research, Hampstead.

Alkaline extract. Prepared according to Burn & Ling [1933].

Acid extract. Prepared according to Best & Campbell [1936].

Thyrotropic extract. Prepared by Dr F. G. Young and found by Marks & Young [1938] to possess fatty liver-producing activity in rabbits.

Adrenaline-free extract of ox adrenal. Prepared by Dr F. G. Young.

Estimation of ketone bodies in the blood

Several methods are available for the estimation of ketone bodies in blood, but choice is limited by the small quantities of blood available in the type of experiment to be described. Thus the Denigès-Van Slyke method is useless owing to its insensitivity. The Behre and Benedict colorimetric method even in the micro-modification of Cantoni [1935] is still not sufficiently sensitive. We are thus left with the more sensitive but much less specific iodimetric method. The determination of the small difference in acetone to be measured is on safer grounds since a titrimetric procedure is used. The Engfeld-Pincussen [1928] method is entirely satisfactory. In the experiments using rabbits, both the Cantoni and Engfeld-Pincussen methods were employed.

Estimation of ketone bodies in the urine

The choice of method here is simpler. The amount of ketone bodies excreted daily by rats is so variable that increases of over 200–300 % alone can be regarded as significant. The actual amount excreted daily by three rats on a fat

diet gives 1–6 mg. precipitate with the Denigès-Van Slyke reagent, so that this method is unquestionably the most suitable.

Estimation of liver fat

The method of Leathes and Raper [1925] was followed in all essential details.

Methods. II. Biological

Rats. These were all of Wistar strain and were killed for estimation of liver fat by a blow on the head. The urinary output of a single rat is very small and this is particularly the case when anterior pituitary extracts have been administered. Rats were therefore put in groups of three in each metabolism cage and analyses carried out on the mixed urine of the group, the urine being preserved under toluene. In this way, individual variations of the rats were partly eliminated, but even so there still persisted a great variation in the ketone body excretions of different groups of rats treated under identical conditions (see Fig. 1).

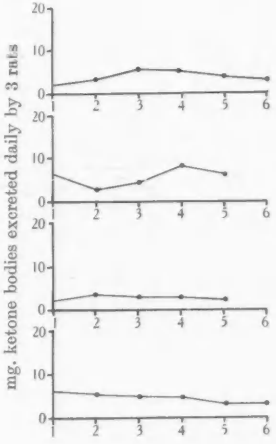

Fig. 1.

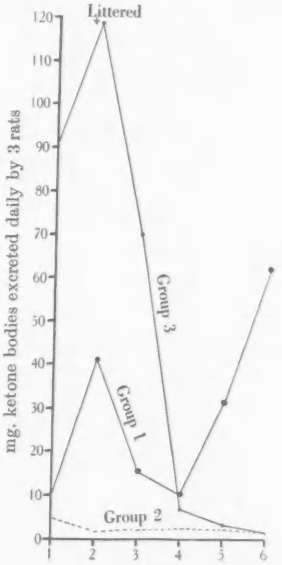

Fig. 2.

Fig. 1. Ketonuria of 4 groups of rats fed on a 75% butter-fat diet. July, 1937.

Fig. 2. Ketonuria of 3 groups of 3 rats all fed on a butter-fat diet. Group 1, July 1937, 97% fat. Group 2, Sept. 1937, 97% fat. Group 3, July 1937, 75% fat, included one pregnant rat.

Because of the marked sex variation in the ketonuria after injection of pituitary extracts each group of rats should be exclusively of the same sex. Pregnant animals must be excluded since pregnancy greatly increases the ketosis of rats on a fat diet [Burn & Ling, 1933]. This is well shown in Fig. 2,

which also confirms the findings of these workers in showing a very marked seasonal variation in the ketonuria of rats fed on a high fat diet.

The early part of this work was carried out using rats fed on a mixture of 97 % fat and 3 % salts, but it was soon realized that rats varied a great deal in the amount of this diet which they ate so that it was uncertain whether they were on a high fat diet or were partly or even wholly starving. It was therefore considered preferable to starve them over a short period rather than maintain them doubtfully on a fat diet.

All injections were administered subcutaneously.

Rabbits. A single rabbit was used in each experiment, bleeding from the marginal vein being effected without the use of irritants.

Results

Rabbits. Rabbits were injected with various types of anterior pituitary extract, and the blood ketones estimated by the Cantoni method hourly for 5 hr. after the injection. Of 68 rabbits 22 showed slight increases of blood ketones detectable by this method of determination.

In a further series of 12 rabbits similarly injected with various types of extract the blood ketones were determined by the Engfeld-Pincussen method at the time of the injection and 3 and 5 hr. after. In each case a definite rise in β-hydroxybutyric acid content of the blood occurred and in 4 the concentration 5 hr. after the injection exceeded 22 mg. per 100 ml. of blood, and was therefore not accurately measurable without modification of the technique. The normal value varied from 3·6 to 9·0 mg. per 100 ml. of blood. In the other 8 rabbits there were increases varying from 0·9 to 12·0 mg./100 ml.

It therefore appears that of the first group of 68, in which ketones were estimated by the Cantoni method, 22 presumably showed a considerable rise in blood ketones while the remaining 46 may have shown rises not detectable by this method. The fact that all rabbits in the second series exhibited rises in ketone bodies as measured iodimetrically suggests that this was the case. The blood ketones of 6 of these 12 rabbits were estimated by both methods and rises in blood ketones were detectable by the Cantoni method when they were very great as determined iodimetrically.

The determination of ketogenic activity using rabbits and determining the blood ketones by the Engfeld-Pincussen method was tedious and lengthy as it was considered necessary to use at least 12 rabbits for investigating an extract of unknown ketogenic activity.

Rats. The early part of this work was carried out on rats fed on a diet of 97 % butter-fat and 3 % salt mixture. Fig. 3 shows that over 15 days a control group of three rats excreted between 1·2 and 7·5 mg. daily of "total ketone bodies" expressed as acetone. Four injections of a saline extract of anterior pituitary into each rat of the experimental groups produced rises in ketonuria above the level previously existent and two of these rises were above the maximum level attained during the whole period of experiment by the control group. Injection of neutralized alkaline extract also produced an increase in ketonuria with a dose of 2 ml.

Definite conclusions from this experiment must be accepted with reserve, since it is open to two criticisms: firstly, no attempt had been made to segregate the sexes and secondly, repeated injections were made into the same animals. However in general this experiment confirmed the ketogenic activity of anterior pituitary extracts.

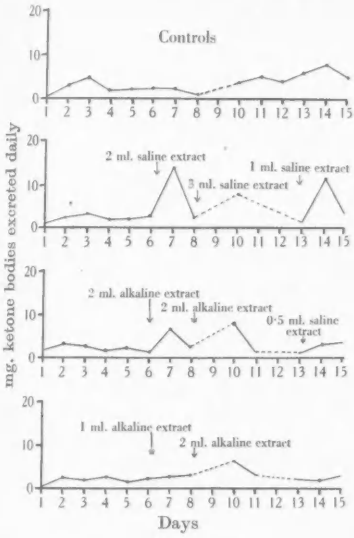

Fig. 3.

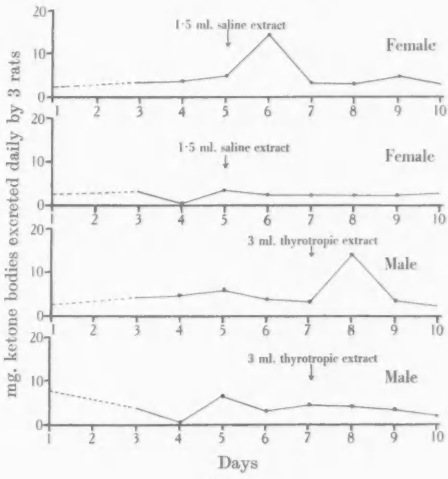

Fig. 4.

Fig. 3. Ketonuria of 4 groups of 3 rats fed 97% fat, Sept. 1937. 3 groups received anterior pituitary extract.

Fig. 4. Effect of anterior pituitary extracts on rats fed on a 97% fat diet, Oct. 1937.

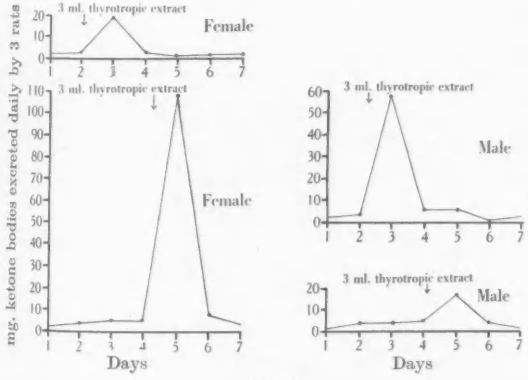

Fig. 5.

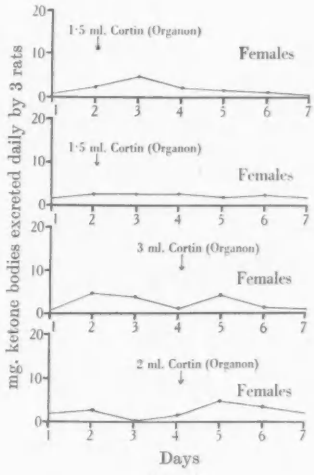

Fig. 6.

Fig. 5. Ketogenic activity of extracts of the anterior pituitary rats fed on a 97% fat diet, Nov. 1937, cross-control.

Fig. 6. Effect of cortin (organon) on ketonuria of rats fed on a 97% fat diet, Nov. 1937.

An attempt to obtain a reliable test of ketogenic activity was made by maintaining four groups of three rats each on a 97 % fat diet over 10 days. Two groups were male and two female. On the 5th day one male and one female group were injected and on the 7th day the others were injected. In this way the uninjected rats acted as controls at the time when the others were injected and the former were later injected with the others as controls. Fig. 4 illustrates the results of this experiment and confirms the finding that female rats are more sensitive than male rats under these conditions to the ketogenic factor in these extracts.

It appeared possible that the time of experiment could be cut down, and accordingly four similar groups were given a fat diet for 7 days, with injections on the 2nd and 4th days with the same system of cross-control as before. Fig. 5 shows that the increases in ketonuria following an injection of thyrotropic extract were greater than in the previous experiments. Again the average response by females was greater than that by males, although one group of males showed a greater response than did one group of females. A sample of Cortin (Organon) was investigated in this way for ketogenic activity with the negative results shown in Fig. 6. This experiment formed an excellent control in demonstrating that ketogenesis is not stimulated by a non-specific tissue extract.

In all of these experiments, rats of Wistar strain had been used and as the supply was limited an attempt was made to employ ordinary stock rats but these produced a more variable ketonuria than those of Wistar strain. In two groups the ketonuria reached 33 and 11 mg. daily per group before any injection had been given. In all succeeding experiments Wistar rats were employed.

Preliminary experiments using rats under fasting conditions gave promising results although there was still some variation in the responses of the four groups. Best & Campbell [1936] have shown that female rats given daily subcutaneous doses of anterior pituitary extract during a 3-day fast show marked increase of liver fat as well as greatly augmented ketonuria. It appeared possible that by administering an active extract more frequently during a shorter period of fasting the onset of these effects might be accelerated.

Actually three groups of rats were fasted overnight; one group was used as control, another given injections at 8 a.m., 4 p.m., 12 midnight and 8 a.m. the following morning and the remaining group given a single injection of extract at 8 a.m. on the first morning after the preliminary fast of 12 hr. Urine was analysed for ketone bodies every 8 hr. Table I, exp. 1 shows that under these conditions the control groups produced no ketonuria, but those receiving a single injection showed a ketonuria commencing between 8 and 16 hr. after the injection and reaching a maximum of 2·7 mg. for the 8-hr. period ending 24 hr. after the injection. The liver fat of these animals averaged 0·36 g./100 g. rat as compared with the controls which had 0·16 g./100 g. rat. Those receiving repeated doses of extract similarly showed a ketonuria commencing between 8 and 16 hr. after the first injection and steadily rising to 4·8 mg. at the end of the experiment. The liver fat of these averaged 0·65 g./100 g. rat. Table I also shows results of further experiments on these lines. Exp. 3 suggests that the ketogenic response of males under these conditions is greater than that of females. In exp. 2 and 3 the urinary volumes were increased by giving each rat 6 ml. of water by stomach tube every 8 hr.

The ketonuria following a single injection in females was very small, but the increases in liver fat very definite, so the experiments were repeated with a single dose of extract and modified in such a way that the relationship between ketonuria and liver fat could be followed. The rats were fasted overnight and, with the exception of the controls, given an injection of an active extract. Groups

Table I. *Ketonuria and liver fat following thyrotropic extract of the anterior pituitary administered after 12 hr. fasting*

Series I. No injection.
Series II. Four injections of thyrotropic extract at 8-hr intervals.
Series III. A single injection of thyrotropic extract at commencement of the experiment.
All injections consisted of 3 ml. of extract from one anterior lobe.

Series	Rat	Wt. g.	Liver fat g./100 g. rat		Ketonuria mg./3 rats/8 hr. 0–8 hr.	8–16 hr.	16–24 hr .	24–30 hr
			Females					
I	1	250	0·17					
	2	230	0·13	Av. = 0·16	0·0	0·0	0·0	0·0
	3	215	0·19					
II	4	245	0·68					
	5	200	0·65	Av. = 0·65	0·0	2·5	3·4	4·8
	6	200	0·61					
III	7	260	0·17					
	8	200	0·50	Av. = 0·36	0·0	1·7	2·7	0·6
	9	225	0·42					
			Females					
I	1	215	—					
	2	205	0·21	Av. = 0·19	0·0	0·0	0·0	0·0
	3	220	0·17					
II	4	240	0·60					
	5	220	0·47	Av. = 0·55	0·3	5·0	8·8	16·0
	6	195	0·57					
III	7	190	0·42					
	8	200	0·28	Av. = 0·32	0·0	0·8	0·4	0·0
	9	200	0·27					
			Males					
III	1	200	0·22					
	2	200	0·40	Av. = 0·29	31·4	128·6	126·1	13·7
	3	230	0·25					

In this series using male rats only three injections were given during the experimental period.

of rats were killed every 4 hr., the controls being killed simultaneously with the 12-hr. group. All surviving rats except the controls were given 6 ml. of water by stomach tube every 4 hr., in order that the urinary volumes should be adequate. In this way, urinary ketones and liver fats were determined 4, 8 and 12 hr. after injection. Controls were not killed at 4 and 8 hr. since any changes would be still more obvious at 12 hr. Table II illustrates the results obtained in such experiments. In exp. 1 with male rats there was practically no ketonuria, while the liver fat increased from an average control level of 0·093–0·34 g./100 g. rat after 4 hr. and to 0·29 g./100 g. rat after 8 hr. Unfortunately only one figure for the liver fat of the group at 12 hr. was obtained. In exp. 2 of Table II a ketonuria occurred beginning during the first 4 hr. after the injection and rising to 36·2 mg. for the whole period of experiment, while the liver fat showed no significant change. In exp. 2 of Table II rats were not killed at 4 hr. after the injection. In a further experiment in which an accidentally inactivated thyrotropic extract was used there was no ketonuria and no significant change in liver fat.

It was considered possible that by increasing the duration of the previous fast more consistent results might be obtained and therefore the experiment was redesigned. The total period of fasting was 48 hr. and began at 9 p.m.; 24 hr. later the 12 rats were transferred to 4 Hopkins metabolism cages, 3 to each cage.

Table II. *Ketonuria and liver fat following thyrotropic extract of the anterior pituitary administered after 12 hr. fasting*

All the injected animals received 3 ml. of extract from one anterior lobe.

	Rat	Wt. g.	Liver fat g./100 g. rat		Mg. ketones/ 3 rats
		Exp. 1. Males			
Control	1	200	0·095		
	2	195	0·092	Av. = 0·093	0·0
	3	205	0·093		
4 hr.	4	190	0·49		
	5	220	0·25	Av. = 0·34	0·1
	6	190	0·28		
8 hr.	7	215	0·30		
	8	240	0·25	Av. = 0·29	0·0
	9	200	0·32		
12 hr.	10	215	—		
	11	200	0·14		0·0
	12	200	—		
		Exp. 2. Males			
Control	1	125	0·17		
	2	135	0·12	Av. = 0·13	0·0
	3	125	0·11		
4 hr.	4	—	—		
	5	—	—		1·8
	6	—	—		
8 hr.	7	160	0·13		
	8	110	0·15	Av. = 0·14	9·5
	9	150	0·14		
12 hr.	10	175	0·16		
	11	125	0·23	Av. = 0·20	36·2
	12	140	0·22		

The urine passed by all groups for the next 12 hr. was collected, pooled and analysed for ketones. At 9 a.m., 12 hr. after the rats had been transferred to the cages, injections of extract subcutaneously and 6 ml. of water by stomach tube were given to all rats of the first three groups; 4 hr. later a group of rats was killed and their liver fats and excreted ketones determined. The remainder of the injected rats were given 6 ml. more of water by stomach tube and 4 hr. later another group of rats was killed. The remaining three injected rats were again given 6 ml. of water and 4 hr. later, at 9 p.m., all the surviving rats were killed. In this way the ketone bodies excreted and the liver fats could be determined 4, 8 and 12 hr. after the injection.

The results of this type of experiment were very satisfactory. With male rats there was marked ketonuria beginning during the first 4 hr. after the injection and steadily rising until the end of the experiment, the controls excreting no ketones. The liver fats were appreciably increased 8 hr. after the injection and showed a still further rise at 12 hr. after the injection. This is clearly shown in Tables III and IV. It is impossible to determine whether there is an increase in the liver fat after 4 hr. without employing a very much larger number of animals.

Such experiments carried out on female rats were less satisfactory in that there was a much less marked ketonuria with greater increases of liver fat. The relationship of this to the findings of other workers will be discussed later.

Table III. *Ketonuria and liver fat following saline extract of the anterior pituitary administered after 36 hr. fasting*

All the injected animals received 2 ml. of extract from one anterior lobe.

	Rat	Wt. g.	Liver fat g./100 g. rat		Mg. ketones/ 3 rats
		Exp. 1. Males			
Control	1	155	0·15		
	2	150	0·19	Av. = 0·17	0·0
	3	145	0·17		
4 hr.	4	175	0·23		
	5	175	0·18	Av. = 0·19	1·8
	6	160	0·16		
8 hr.	7	195	0·26		
	8	155	0·28	Av. = 0·26	24·8
	9	160	0·24		
12 hr.	10	160	0·30		
	11	150	0·32	Av. = 0·30	108·0
	12	140	0·29		
		Exp. 2. Males			
Control	1	160	0·13		
	2	155	0·19	Av. = 0·16	0·7
	3	145	0·17		
4 hr.	4	155	0·10		
	5	185	0·14	Av. = 0·13	1·0
	6	185	0·14		
8 hr.	7	160	0·23		
	8	150	0·27	Av. = 0·23	22·3
	9	185	0·20		
12 hr.	10	180	0·24		
	11	175	0·25	Av. = 0·24	182·0
	12	155	0·23		
		Exp. 3. Females			
Control	1	175	0·22		
	2	170	0·21	Av. = 0·22	0·0
	3	170	0·22		
4 hr.	4	185	0·21		
	5	160	0·21	Av. = 0·19	0·0
	6	180	0·16		
8 hr.	7	170	0·24		
	8	220	0·27	Av. = 0·27	6·8
	9	155	0·31		
12 hr.	10	180	0·40		
	11	170	0·36	Av. = 0·38	9·1
	12	200	0·39		
		Exp. 4. Females			
Control	1	150	0·19		
	2	165	0·27	Av. = 0·23	0·0
	3	160	—		
4 hr.	4	135	0·26		
	5	170	0·26	Av. = 0·28	0·0
	6	155	0·32		
8 hr.	7	175	—		
	8	165	0·26	Av. = 0·23	3·0
	9	165	0·18		
12 hr.	10	155	0·23		
	11	135	0·40	Av. = 0·34	10·2
	12	135	0·40		

Table III (*cont.*)

	Rat	Wt. g.	Liver fat g./100 g. rat		Mg. ketones/ 3 rats
		Exp. 5. Females			
Control	1	165	0·18	Av. = 0·17	0·0
	2	150	0·17		
	3	160	0·16		
4 hr.	4	150	0·23	Av. = 0·25	0·0
	5	165	0·30		
	6	175	0·22		
8 hr.	7	135	0·37	Av. = 0·30	1·3
	8	160	0·24		
	9	160	—		
12 hr.	10	160	0·34	Av. = 0·35	6·3
	11	150	0·37		
	12	160	0·34		

Table IV. *Ketonuria and liver fat following thyrotropic extract of the anterior pituitary. Male rats after 36 hr. fasting*

All the injected animals received 3 ml. of extract from one anterior lobe.

	Rat	Wt. g.	Liver fat g./100 g. rat		Mg. ketones/ 3 rats
Control	1	125	0·15	Av. = 0·14	0·0
	2	135	0·12		
	3	125	0·16		
4 hr.	4	—	—		3·5
	5	—	—		
	6	—	—		
8 hr.	7	165	—	Av. = 0·16	36·0
	8	140	0·14		
	9	165	0·18		
12 hr.	10	135	0·18	Av. = 0·21	100·0
	11	125	0·21		
	12	120	0·24		

Table V shows an experiment carried out using an adrenaline-free extract of ox adrenal. There was no ketonuria and no increase in liver fat and this experiment together with that shown in Fig. 6, forms an excellent control in showing that ketogenesis is not stimulated by non-specific tissue extracts.

Discussion

From the results it seems preferable to use fasting rats for the determination of ketogenic activity of extracts of endocrine organs, rather than fat-fed animals as the former give more consistent results. Black *et al.* [1934] similarly preferred to study the influence of pituitary extracts on the ketone body excretion of the fasting animal, thereby obtaining consistently low control values. Butts *et al.* [1934] also found regular responses in fasting rats.

The sex variations in the responses of the rats are difficult to explain. Burn & Ling [1933] found no increased ketonuria in fat-fed male rats given anterior pituitary extracts. Best & Campbell [1936] employed only female rats and do not refer to results obtained in males; more recently [1938] they say "the production of ketone bodies (due both to fasting and to fasting with the administration of anterior pituitary extract) is greater in female guinea-pigs than in males". However these differences were slight. Deuel *et al.* [1937] find that

Table V. *Ketonuria and liver fat following an extract of the adrenal administered after 36 hr. fasting*

All injected animals received 3 ml. of extract.

	Rat	Wt. g.	Liver fat g./100 g. rat		Mg. ketones/ 3 rats
		Exp. 1. Females			
Control	1	125	0·20		
	2	180	0·14	Av. = 0·17	0·0
	3	130	0·18		
4 hr.	4	95	0·16		
	5	110	0·14	Av. = 0·15	0·0
	6	95	0·14		
8 hr.	7	125	0·18		
	8	140	0·19	Av. = 0·16	0·0
	9	155	0·12		
12 hr.	10	115	0·12		
	11	115	0·16	Av. = 0·15	0·0
	12	105	0·17		
		Exp. 2. Females			
Control	1	75	0·12		
	2	75	0·17	Av. = 0·13	0·0
	3	90	0·11		
4 hr.	4	175	0·11		
	5	140	0·14	Av. = 0·14	0·0
	6	120	0·17		
8 hr.	7	135	0·22		
	8	115	0·23	Av. = 0·25	0·0
	9	95	0·31		
12 hr.	10	80	0·09		
	11	90	0·14	Av. = 0·12	0·0
	12	105	0·14		

female rats consistently have a higher level of ketonuria than males during a fast following the administration of a high fat diet. The earlier findings described in this paper are in agreement with this, and indicate that on a high fat diet female rats exhibit a greater increase in ketonuria following the administration of extract of the anterior pituitary than males. The results of experiments using fasting rats in which males exhibit a greater ketonuria than females in response to anterior pituitary extracts are of great interest in view of the general agreement that in fat-fed rats experimental ketonuria is more easily induced in female than in male animals. Butts *et al.* [1934] found that fasting male and female rats gave equal ketogenic responses to anterior pituitary extracts. It would be unwise to speculate on this sex difference in the light of present knowledge.

Similarly, it is unwise to speculate on the cause of the enormous ketosis occurring in the pregnant rat on a high fat diet. That this is not peculiar to the rat has been shown by Bullock [1930] who finds that pregnant women pass acetone in their urine much more readily than non-pregnant women do, a finding which has been confirmed by the present author (unpublished observation).

Geelmuyden [1920] believes that the sequence of events in the production of ketones is:

Fat migration → fatty liver → ketosis.

Dible [1932] observes that in fasting rats "an animal whose urine gave a rapid and intense acetone reaction by the nitroprusside method at the time of

killing could be confidently expected to show a high fat content of the liver with an abundance of depot fat". Gray [1937], referring to infantile vomiting, observed that cases with high ketonaemia showed at autopsy marked hepatic fatty change. Best & Campbell [1938] believe that it is probable that the rise in liver fat precedes the increase in ketonuria.

The experiments of Table III show clearly that in female rats the increase in liver fat precedes the ketonuria when anterior pituitary extracts are administered after 36 hr. fasting; the rise in liver fat commences within 4 hr. of the injection. This is in agreement with Best and Campbell who find that the increase in liver fat occurs within 24 hr. of the injection and with those of Steppuhn [1934] who reports an increase in liver fat within 4 hr.

On the other hand, in male rats there was much greater ketonuria and much less increase in liver fat than in females. In males the ketonuria began within 4 hr. of the administration of the extract, but it is difficult to assess the time of onset of the increases in liver fat as they were so small.

On considering all the results obtained irrespective of sex those animals producing the greater ketonuria showed smaller increases in liver fat, and vice versa. If one considers that the ketonuria occurs at the expense of the fat brought to the liver, then clearly the relationship of the times of onset of ketosis and liver change cannot be rigid. The amount of liver fat must depend on the balance between fat mobilized from the depots and that catabolized by the liver. If fat catabolism can occur at a greater rate than fat can be brought to the liver, then clearly ketonuria will be more marked than and may precede the increase in liver fat. If, on the other hand, fat mobilization to the liver occurs more rapidly than that organ can catabolize it then the increase in liver fat may be more marked than the ketonuria and the sequence of events will depend on the relative rates of mobilization and catabolism.

These findings throw no light on the identity or otherwise of the ketogenic and "fatty liver-producing" factors of the anterior pituitary. It is therefore essential to determine the two activities simultaneously in any investigation of these actions of extracts of endocrine organs.

Summary

1. The results of previous workers in regard to seasonal, sex and pregnancy variations of ketonuria in rats on a high fat diet are confirmed.

2. The ketogenic action of extracts of the anterior pituitary gland on fasting and fat-fed rats, as well as on fasting rabbits, has been confirmed.

3. Fasting rats give more consistent responses to ketogenic extracts than either fat-fed rats or fasting rabbits.

4. Ketogenic response to such extracts is greater in fasting male rats than in fasting female rats, while the increases in liver fat are greater in females.

5. After 36 hr. fasting, the administration of a ketogenic preparation to female rats resulted in a ketonuria which followed the increase in liver fat. In male rats under similar conditions the resulting increase in liver fat was so small that it was not possible to determine the time of onset in relationship to the commencement of ketonuria.

6. Evidence is presented suggesting that a large ketogenic response to anterior pituitary extracts is accompanied by a small increase in liver fat, and conversely; and that the effect on ketonuria should be studied together with that on liver fat. A test in which this may be done is described.

The expenses of this investigation were defrayed partly by a grant from the Medical Research Council and partly by a grant from the Graham Fund Committee of the University of London to both of which bodies my thanks are due. I have also to express my thanks to Dr F. G. Young of the National Institute for Medical Research for supplying many of the extracts used and also for much helpful criticism.

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CII. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES

IV. THE PERMEABILITY OF "GHOSTS" TO CATIONS

By HUGH DAVSON[1] and ERIC PONDER

From the Department of Physiology, Pharmacology, and Biochemistry, University College, London, and the Biological Laboratory, Cold Spring Harbor, N.Y.

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The cellular residue, obtained by centrifuging a haemolysed suspension of the red cells of certain species, e.g. the rabbit (the erythrocyte "ghosts"), forms the basis of the present study, which is concerned with the extent to which the erythrocyte is able to maintain its integrity as a cell after it has passed through the phase of permeability to the large molecule of haemoglobin.

The work of Fricke & Curtis [1935], who showed that the red cells of the rabbit etc. may exhibit very similar electrical impedance both before and after haemolysis, suggests the possibility that the normal impermeability to cations, which is the most characteristic property of the red cell, may be maintained, at least in part, after the escape of haemoglobin. The observation of Ponder & Marsland [1935] that during saponin lysis the pigment may escape from the red cell at a rate very much less than that required for diffusion through an aqueous medium, further indicates that during the actual escape of haemoglobin the red cell membrane still presents certain structural impediments to the free passage of this molecule. Further evidence of the at least partial maintenance of the original structure is provided by the microscopical appearance of the ghost after "reversal of haemolysis", for the form is more or less that of the typical bi-concave disk, although it is true that the disk-sphere shape transformation cannot be effected either by enclosure between slide and coverglass, or by addition of lecithin.[2]

One of the most striking properties of the erythrocyte is its cation impermeability, which may be used as an index of the degree to which the cell membrane is altered during and after the escape of haemoglobin. In this paper the permeability of the ghost to cations is studied by direct chemical methods.

Experimental

The essential of the procedure is to suspend a quantity of ghosts containing a known amount of K^+ or Na^+ in a given quantity of isotonic NaCl or KCl, and to determine the K^+ or Na^+ in them after centrifuging. The amount of cation found in the ghosts after this treatment is compared with that required for an equal distribution of the cell cation between the ghosts and the external medium. For the K^+ studies the ghosts were formed by addition of rabbit blood to 0·04 *M* KCl, centrifuging and washing the cells in 0·16 *M* KCl; by preparing them in this way one can be sure that they will contain appreciable quantities of K^+. The washing with 0·16 *M* KCl causes the so-called "reversal" of haemolysis, whereby the ghosts become impermeable to haemoglobin. 0·5 ml.

[1] Beit Memorial Fellow.

[2] One of us will shortly publish an account of the shape changes which occur in the case of the ghosts produced by hypotonic lysis.

of the concentrated suspension is added to varying amounts of 0·16 *M* NaCl and after the appropriate interval centrifuged down, the supernatant fluid is removed and the K^+ is determined in the cells as described by Davson [1934]. For studies of Na^+ penetration, the haemolysis was brought about in 0·04 *M* NaCl and the subsequent "reversal" produced with 0·16 *M* NaCl; the ghosts were then suspended in 0·16 *M* KCl. By using graduated centrifuge tubes an approximate estimate of the volume of cells, whose cation content is determined, may be obtained. The amount of cation in the original cell suspension is determined separately.

Results

A number of preliminary experiments using a rather simpler technique than that described above gave ample proof that the ghosts are quite permeable to cations, it being quite possible to wash out all the K^+ with two successive washings with NaCl, so that the only problem which remained was to show whether the time required to reach diffusion equilibrium were greater than that necessary for manipulating the cells (6–10 min.) or not. In Table I, Exps. 12, 13 and 15 were performed in the manner described above; they show first that there is a very rapid loss of Na^+ and K^+ and secondly that this loss approximates fairly closely to that required for diffusion equilibrium; the general trend of these experiments and of a number of others not shown, however, appeared to indicate a greater approximation to equal distribution of cation between the two phases after the lapse of 15 min. than when the interval was smaller (by "0 min." is meant the minimum time it was possible to keep the cells in contact with the suspension medium). As the errors in this technique are large compared with those in erythrocyte studies, owing chiefly

Table I

Exp. no.	Dilution. Cells: suspension medium	Initial concentration	Final concentration	Theoretical final concentration	Time min.	Temp. °C.
12 (Na^+)	1 : 5	100	16·5	14·5	0	20
	1 : 9	100	8·9	8·9	0	
	1 : 21	100	4·5	3·3	0	
13 (Na^+)	1 : 21	100	3·0	2·4	0	20
	1 : 21	100	2·0	2·4	15	
	1 : 21	100	2·0	2·4	60	
15 (K^+)	1 : 5	100	12·6	11·1	0	20
	1 : 9	100	7·5	4·9	0	
	1 : 21	100	2·5	2·4	0	
	1 : 5	100	12·0	12·4	15	
	1 : 9	100	7·3	7·7	15	
	1 : 21	100	2·5	3·0	15	
17 (K^+)	1 : 9	—	106	100	0	20
	1 : 9	—	106	100	15	
	1 : 11	—	104	100	0	
	1 : 11	—	108	100	15	
20 (K^+)	1 : 6	—	101	100	0	0
	1 : 6	—	97	100	2	
	1 : 6	—	97	100	10	
	1 : 6	—	102	100	30	
22 (K^+)	1 : 6	—	106	100	0	0
	1 : 6	—	107	100	0	
	1 : 6	—	102	100	20	
	1 : 6	—	105	100	20	

to the difficulty in sedimenting the ghosts satisfactorily and the small amounts of cation left in the cells for analysis, it must be conceded that a systematic error in the direction of a decreasing concentration of cation in the cells with time might easily be involved. To make quite sure of this point a set of experiments was carried out in which the K^+ contents of both the suspension medium and the cells, equal amounts of which being measured out in micro-pipettes, were deter-

mined directly. This proved to be an improvement on the technique described earlier chiefly because the necessity for centrifuging the cells down completely is not so great, and the use of micro-pipettes gives an accurate estimate of the volume of material used for analysis.

The results of some of the experiments done in this manner are shown in the same table (Exps. 17, 20 and 22). The value of the concentration of K^+ in the supernatant fluid is put at 100 in the "Theoretical column" and the amount of K^+ in an equal volume of cells is shown in the "Final concentration" column as a percentage of the theoretical. It must be concluded from these results that the time taken to achieve diffusion equilibrium is less than that required for the manipulation of the cells, even when the temperature is kept as low as 0°. The failure to obtain equal quantities of K^+ in cells and supernatant fluid, manifest particularly in Exps. 17 and 22, is most probably due to the presence of unhaemolysed cells in the ghost preparation, a difficulty it has been found almost impossible to avoid. It should perhaps be mentioned that in one experiment at 0° values for the cell concentration of K^+ of 157, 130, 114 and 118 after intervals of 0, 2, 5 and 15 min. respectively were obtained. As it has been found impossible to repeat such a result, it must be concluded either that the ghost preparation was unusually resistant to the migration of cations or that it contained an unusually large number of unhaemolysed cells, and that the decrease in apparent K^+ concentration with time represented fortuitous variations in the quantities of these cells sucked up in the pipettes.

Discussion

1. *Electrical properties.* The experimental section of this paper indicates that the erythrocyte ghost is rapidly permeable to cations; the rate of migration may be, nevertheless, very much less than that which would occur were the cation diffusing through an aqueous medium. The fact that the ghosts retain the electrical properties of their parent erythrocytes, although somewhat modified, is not necessarily at variance with this result because of the way in which the conductivity data are obtained. In the conductivity method [Fricke & Curtis, 1935] the resistances of the suspension of ghosts and of the suspension medium are measured, and the conductivity of the ghosts can then be calculated provided that their volume concentration is known. The volume concentration may be found, somewhat roughly, by use of the high speed haematocrite, or it may be assumed to have a certain value, as in Fricke & Curtis' investigation; in either case, the figure for the volume concentration is subject to some error. If this error is 10%, say, then the calculation will give the ghosts a conductivity amounting to at least 10% of that of the suspension medium. All that Fricke & Curtis showed was that the conductivity of the ghosts could not be greater than 10% of that of the surrounding fluid, and that it was probably very much less; indeed, their experimental results were compatible with its being zero, when all possible sources of error were taken into account. But the results are also compatible with the ghosts' having a small conductivity, of the order of a few % of that of the suspension medium corresponding to a rate of cationic diffusion which might be very much greater than that observed here. Thus the present paper serves to emphasize what Curtis [1936] seems willing to admit, namely that the electrical properties of cell suspensions determined by present methods are a poor index to the actual permeability of the cell membrane.

2. *Reversal of haemolysis.* The problem of the essential difference between the intact erythrocyte and the haemolysed ghost has now to be discussed, and at first the discussion will be confined to the ghost formed after hypotonic haemolysis. If the erythrocyte in hypotonic saline swells up so that its envelope is subjected to strain, the envelope allows the passage of haemoglobin and of course of the cations Na^+ and K^+; in consequence of this interchange further strain on the membrane is avoided, and if the latter is not elastic, it will tend to

remain in its stretched state.[1] Washing the ghost with further quantities of hypotonic saline will then wash out further quantities of haemoglobin; if, on the other hand, a stronger saline is used, e.g. 0·16 *M* NaCl, the contraction of the ghost due to the osmosis of water is sufficient to restore the ghost to a haemoglobin-impermeable cell, and further washing with the stronger saline causes no loss of the haemoglobin remaining inside. The results of the present work show that the ghost in this restored state is still permeable to cations, although the rate of exchange may be slow compared with what it was during the actual escape of haemoglobin.

The ghost in the restored state after osmotic haemolysis is certainly an unstable system, since it still contains some haemoglobin, and is separated from an aqueous salt solution by a salt-permeable membrane. This point may be demonstrated in a variety of ways, one of which is by measuring the conductivity of ghost suspensions after reversal of haemolysis. To 1 ml. of washed rabbit cells, contained in a small conductivity cell, are added 4 ml. of water, and a few minutes later 1 ml. of 5 % NaCl (this produces the reversal of haemolysis), and conductivity measurements at 1000 cycles are made minute by minute (Table II). The slow increase in resistance of the cell suspension is best accounted for on the basis of a slow increase in volume of the cells, due to the entry of water. This contributes to the re-haemolysis which follows the reversal after a time, and which is characterized by the suspension becoming more and more translucent. Bayliss [1924] explains the re-haemolysis on the basis of the cells being permeable to salts and slowly swelling, to which we would add that the translucence is contributed to by some agglutination.

Table II

Time after reversal, min.	Resistance, ohms
1	102·0
2	104·8
3	106·1
5	108·4
10	111·2
20	112·1
30	113·4

3. *A possible dual mechanism for haemolysis.* While searching for an explanation of the only partial nature of the reversal of haemolysis by hypertonic solutions the following hypothesis was examined, namely that haemolysis occurs as a result of two processes: (*a*) the stretching and rupture of the lipoid envelope producing permeability to cations, and (*b*) the stretching of a protein mesh-work (the stroma protein Boehm [1935]) resulting in permeability to haemoglobin. In the case of osmotic haemolysis, both of these processes occur simultaneously, and we can explain the reversal of haemolysis by the contraction of the protein mesh-work with resulting impermeability to haemoglobin, while permeability to cations remains, as the lipoid layers are irreparably damaged. Where lysis is brought about by lipoid solvents, which presumably would not attack the

[1] It is better to speak of a distorted state instead of a stretched state, for when red cells swell in hypotonic systems, the surface area does not increase measurably, and the cell attains its greater volume by changing its shape from disk to sphere, preserving its surface area unchanged. This has been shown simultaneously and independently by Ponder [1937] and Castle [1937]. One may, however, postulate a very small amount of stretching as a final process, although the amount cannot be measured.

stroma protein, the condition (*b*) is only fulfilled as the result of a secondary process of swelling consequent upon the free permeability to cations caused by the destruction of the lipoid envelope. If this hypothesis is true, then the fading time, i.e. the time required for 90% of the pigment to leave the individual haemolysing cells, of erythrocytes undergoing lysis by chloroform should be of the same order as that for a cell undergoing hypotonic lysis.

Simple visual observation of cells haemolysing in systems containing chloroform, ether, amyl alcohol and other lipoid solvents showed clearly that the fading time was in the neighbourhood of 5 sec., very much longer than the 0·6–0·8 sec. given by Ponder & Marsland [1935] for osmotic haemolysis.

This contradiction would disappear, however, if it could be shown that the permeability of the red cell to haemoglobin produced by hypotonicity were not the "all-or-none" process it has hitherto been considered to be [*v.* e.g. Ponder & Marsland, 1935], but rather that varying states of permeability may be induced by different degrees of stretching. Thus if this were true the fading times obtained by Ponder & Marsland for osmotic haemolysis would be minimal ones, since their hypotonic systems were prepared by micro-injection of very hypotonic solutions, or even of water itself, into droplets of red cell suspensions; the resulting tonicity was accordingly very low, and the swelling of the cells very rapid. Under the conditions of a Donnan swelling the process would necessarily be much slower and so experiments were carried out in which the osmotic pressure differences were controlled so that the rate of swelling was slow. To do this, use was made of the fact that the osmotic pressure within the red cell decreases with increase of temperature, so that cells which are swollen almost to their critical volume at 37° swell further when the temperature is lowered to 25°, say, and, reaching their critical volume, haemolyse. By trial, a tonicity of NaCl was found in which a rabbit red cell suspension would remain intact on a warm microscope stage at 37° but would undergo partial lysis at room temperature, and the temperature of the warm stage was allowed to drop while the cells were being observed. Under these conditions of very slow swelling, the fading times were found to be very much larger than in Ponder & Marsland's systems, and to average from 5 to 10 sec.[1]

Thus the interesting fact that haemoglobin may escape at different rates from the red cell during hypotonic swelling according as the conditions of swelling are varied is brought to light by this investigation; consequently hypotonic haemolysis must not necessarily be considered as a complete rupture of the erythrocyte membrane, but must be provisionally regarded as a state of permeability to haemoglobin, due to a mechanical deformation of the membrane.

The dual theory of haemolysis put forward above, is thus not at variance with the facts so far described. That it is nevertheless probably wrong is shown by the following experiment. A suspension of erythrocytes is haemolysed with chloroform, and the ghosts are centrifuged down; after removing the supernatant fluid the ghosts are washed with hypertonic saline and again centrifuged down. If the factor responsible for the impermeability to haemoglobin were merely a contraction of the protein mesh-work, then in these circumstances the ghosts should be impermeable to haemoglobin, and should appear as a bright red layer at the bottom of the centrifuge tube. Such was not the case; washing with

[1] We are not sure whether or not there is a concentration dependence of the fading time in the case of lipoid solvents as with saponin. If these act by dissolving the lipoid layers in an all-or-none fashion, one would expect there to be none. Nevertheless, more concentrated solutions of chloroform, ether etc. seem to give shorter fading times than less concentrated ones. The very laborious cinematographic technique would be required to settle the question unequivocally.

hypertonic saline merely caused the removal of more haemoglobin from the ghosts, so that they appeared as a white layer. Since it is fairly safe to assume that chloroform is haemolytic because it attacks the lipoid constituents of the membrane, the failure to bring about the reversal of haemolysis must be attributed to a partial dissolution of this constituent, and one may state further that the presence of lipoid in the membrane is a necessary condition for impermeability to haemoglobin, and that a shrinkage of the stroma protein is not sufficient of itself to bring about this state. (This argument is not absolutely conclusive, however, as it may be that the osmosis of water from the cell on washing with hypertonic saline was not rapid enough to cause the shrinkage of the stroma protein.)

The facts established above, taken in conjunction with earlier work by Davson [1937] on the loss of K^+ during swelling, show that there is a continuous process of change occurring in the red cell membrane during osmotic haemolysis. Before the critical volume is reached there is a reversible stage of slow cation permeability. Then there comes a stage at which haemoglobin just begins to leak out, with a fading time of about 10 sec., which corresponds to a rate of diffusion from the cell less than 1/250th of that expected on the basis of free diffusion. Finally in very hypotonic solutions, further stretching of the membrane causes a very rapid loss of haemoglobin with a fading time of less than 1 sec., which corresponds to a rate in the neighbourhood of 1/10th of that required for free diffusion through an aqueous medium.

There is an interesting consequence to the observation that the process of lysis occurring in hypotonic solutions may take as long as 10 sec. The cell, while permeable to haemoglobin must necessarily be so permeable to Na^+ and K^+ as to offer no restriction to their migration, owing to the smaller volumes of these ions, so that a Donnan equilibrium appropriate to the diffusibility of both cations and anions should be established within about 1/50th of a sec.[1] from the beginning of the escape of haemoglobin. If this equilibrium is established, a difference of osmotic pressure will be brought into play corresponding to a concentration difference of about 0·01 *M* NaCl. This difference will be accompanied by a rapid diffusion of water into the cell producing further stretching and consequent acceleration of the rate of escape of haemoglobin. It thus seems that such long fading times would be excluded by these considerations; the difficulty can be got over by ascribing the leakage of haemoglobin in the first instance, not to a uniform stretching of pores over the whole surface but rather to the formation of one or more holes, large enough to permit the unrestricted passage of many haemoglobin molecules simultaneously, so that the restraint to the penetration of haemoglobin consists merely in the limited area of these holes in comparison with that of the rest of the surface of the membrane, and not in the energy required for an individual molecule to pass through any particular pore of a size comparable with its own. In this event, the rate of achievement of a Donnan equilibrium will be considerably restricted: thus if the area of the hole is 1/250th of the area of the membrane, the time required for this process will be 250 times greater than if free diffusion were possible all over the surface.

Thus, whereas the evidence from the fading times of cells undergoing hypotonic lysis appeared to indicate that the escape of haemoglobin should be attributed to a permeability to haemoglobin in contradistinction to a complete

[1] This value is obtained by use of Fricke's equation [1934] using a value for D for NaCl of 1×10^{-5} cm.^{2}/sec. It is obviously only approximate.

collapse of the cell membrane, further consideration leads to a conclusion midway between these two extremes, i.e. that the escape of haemoglobin occurs through one or more holes whose total area may vary from 1/10th to 1/250th of that of the area of the cell. If this is true, the diffusion coefficients appropriate to the different fading times of cells undergoing lysis calculated by Fricke [1934] should be interpreted as estimates of the effective areas available for diffusion.

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CIII. THE PHOTOMETRIC ESTIMATION OF COBALT

By HERBERT TAYLOR MACPHERSON and JAMES STEWART

From the Moredun Institute, Animal Diseases Research Association, Gilmerton, Midlothian

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Various colorimetric methods have been employed for the estimation of cobalt in soils, animal organs, etc., notably that of Stare & Elvehjem [1932], recently modified by Kidson *et al.* [1936]. This method depends on the development of a red-coloured complex of nitroso-*R*-salt [$C_{10}H_4.OH.NO.(NaSO_3)_2$] and cobalt, the intensity of the colour being proportional to the amount of cobalt present. These workers used a Lovibond tintometer to measure the final colour and Kidson claims to have obtained reproducible results with as little as 0·0001 mg. cobalt.

In such material as grasses and animal organs in which the cobalt content is minute, a very accurate method of measuring the quantity of cobalt complex is essential. The present work was undertaken primarily to see whether the Pulfrich photometer could be successfully substituted for the tintometer, since an efficient photometer would give considerably more accurate readings. With a tintometer there is no means of compensating for colours, notably yellow and green, developed in the solution by elements other than cobalt. Copper and iron in particular interfere seriously if present in any quantity and must be removed. With the photometer, on the other hand, provided that the appropriate colour filter is used, it is found that removal of copper is not necessary if present in small amount and iron need not be removed at all.

The extinction coefficient, which could be read directly on the instrument, was used as a measurement of the depth of colour of the solution. With pure cobalt solutions it was found that the extinction coefficient was directly proportional to the amount of cobalt present, and very small quantities of cobalt could be accurately estimated, the error being approximately $\pm 0{\cdot}0001$ mg. Any colour filter could be used for these solutions, a convenient range of readings being given by a blue filter (S 47).

The effect of other metals was then investigated. Using a cobalt solution containing varying amounts of iron, readings were obtained which depended on the quantity of iron present and were therefore of no value in estimating the cobalt. It was found, however, that if a green filter (S 53) was substituted for the blue, a constant reading was obtained for a particular cobalt solution, no matter what quantity of iron was present. Iron, therefore, does not interfere if this filter is employed. In an attempt to retain the blue filter for the estimation of cobalt, since it gave considerably higher readings than the green, removal of iron with ether as directed by Kidson *et al.* [1936] was carried out. This was unsatisfactory, since the iron was not completely removed even after several washings. Precipitation of iron as hydroxide by means of ammonia was unsuccessful, since some cobalt was removed even in presence of excess ammonium chloride: the basic acetate method also removed part of the cobalt. A very satisfactory method was found in the precipitation by means of zinc oxide

[Scott, 1917]. In numerous experiments with this method it was found that no cobalt was lost, whilst very complete removal of iron was effected. Using this method of precipitation, under the conditions described later, cobalt can be accurately estimated with either blue or green filters. The green was eventually employed, since it cuts out traces of other elements which may be present.

Since it would be convenient if estimations could be carried out without removal of iron, cobalt solutions containing large amounts of iron were examined. It was found that by a slight modification of Kidson's method, cobalt could be accurately estimated in presence of iron, provided that a green filter was used.

The effect of copper was then investigated. When present in very small amount, copper does not interfere, but if much of it is present, it interferes seriously with the estimation even when the green filter is employed. In quantity, it appears to give rise to a yellowish brown colour in the final solution and must therefore be removed. Precipitation by means of H_2S is satisfactory, if carried out in a solution sufficiently acid to prevent co-precipitation of cobalt. A more convenient and equally effective method of removing the copper is displacement from solution by means of a more electropositive element such as aluminium. The method is described later.

Manganese did not interfere, even in very large amount.

This paper is concerned chiefly with the estimation of cobalt in grasses. The methods, however, could be equally well applied to the analysis of soils, animal organs or blood samples. In none of the normal bloods examined was there any detectable amount of cobalt, but the method would be of use in analysis of blood from cobalt-feeding experiments.

The preliminary treatment of the sample and subsequent estimation of cobalt will be described according to the nature of the substance under examination.

(1) In grasses, neither copper nor iron is, as a rule, present in sufficient quantity to require removal.

(2) In soils, animal organs and bloods, it is generally advisable to remove the considerable amount of iron present, more for convenience in manipulation of the final solution than for any possible interference with the colour development. Since complete removal of iron is not essential in the photometric method, washing with ether in acid solution would also serve, though not so satisfactorily as a precipitation method.

(3) In soils, minerals or other samples containing appreciable amounts of both copper and iron, copper is first removed, either by means of aluminium or precipitation with H_2S, and the iron then precipitated with zinc oxide.

Procedure

5–10 g. of the sample are ashed by igniting for 30 min. at dull red heat. The ash is then taken up with 10–15 ml. concentrated HCl and slowly evaporated, the subsequent procedure depending on whether copper and iron are to be removed. The method of developing the cobalt complex is based on that of Kidson *et al.* [1936].

Reagents required:

HCl: conc., $N/2$ and sp.gr. 1·12 (7·3N).
HNO_2: conc. and 1 : 1.
KOH: 20 % aqueous solution.
Sodium acetate (A.R.).

Zinc oxide (A.R.).
Nitroso-*R*-salt: 0·1 % aqueous solution.
Phenolphthalein: 0·2 % alcoholic solution.
H_2S generator or pure aluminium foil.

(1) When neither copper nor iron is to be removed, as in the case of grasses, evaporate the contents of the basin to complete dryness and take up the residue with 1 ml. HCl (sp.gr. 1·12), 6 drops 1 : 1 HNO_3 and a little hot distilled water. Filter into a 250 ml. conical flask, washing with hot water and bringing the volume to about 25 ml. Boil for 2 min., cool, add 2 ml. nitroso-*R*-salt solution and 2 g. sodium acetate. Heat to about 70°, add 5 drops phenolphthalein and make just alkaline with the KOH solution. Then make just acid with the *N*/2 HCl and boil for 2 min., keeping just acid to phenolphthalein. Add 5 ml. concentrated HNO_3 and boil for a further 2 min. Cool and make up to 25 ml. in a graduated cylinder. Find the extinction coefficient by means of the photometer, using filter S 53 and a 20 mm. cell, with distilled water in the compensating cell. The cobalt present is then found from the standard graph, obtained by plotting the figures given in Table I.

Note. It is advisable to filter all final solutions through a fine-grain paper before taking a reading, since unless they are perfectly clear, a serious error will be introduced. Filtration does not affect the intensity of the colour due to the cobalt.

(2) When iron is to be removed, evaporate the contents of the basin almost to dryness, add 10 drops concentrated HNO_3 and after evaporating almost to dryness, take up the residue with 2 ml. HCl (sp.gr. 1·12) and a little hot water. Filter and wash with a little hot dilute HCl; the volume should be about 25 ml. Boil for 2 min. and cool. Add a slight excess of an aqueous suspension of zinc oxide, boil for 1 min. and filter hot. Wash thoroughly with hot water. Evaporate filtrate and washings to about 20 ml., add 0·5 ml. HCl (1·12) and 6 drops 1 : 1 HNO_3, boil for 2 min. and proceed with the estimation of cobalt as directed above.

Notes. During neutralization with KOH, a precipitate is formed due to the zinc in solution. This, however, does not interfere with the adjustment of the reaction and disappears on addition of the concentrated HNO_3.

Concentrated HNO_3 is preferable to 1 : 1, since without affecting the cobalt complex, it more readily destroys colours formed by other elements, and is necessary in this case to destroy that due to the zinc itself.

(3) When copper is to be removed, evaporate the acid in the basin to about 2 ml., add a little hot distilled water and rinse the contents into a 150 ml. flask. Make the volume up to about 25 ml., warm and saturate with H_2S. Filter and wash with H_2S solution containing HCl.

To remove the copper by means of aluminium, evaporate the contents of the basin almost to dryness, add a little hot distilled water and rinse into a 150 ml. flask, making up to about 25 ml. Add a small piece of pure aluminium foil and heat on the steam bath for 1 hr. The copper, if present in sufficient quantity, forms a spongy deposit on the surface of the metal. The flask should be agitated occasionally to loosen the deposit. Filter and wash carefully with hot water.

In either case, evaporate the filtrate to 20 ml. If no removal of iron is required, proceed with the estimation of cobalt as in (1) above; if iron is to be removed, add 1 ml. HCl (1·12) and 6 drops 1 : 1 HNO_3, boil for 2 min., cool and proceed with addition of zinc oxide as in (2) above. Again all final solutions should be filtered. The cobalt is in all cases found from the standard graph.

It is to be noted that the graph passes through the origin. This shows that there is no interference from any of the other elements present in the solution.

Experimental results

Standard cobalt solution: 0·4034 g. $CoCl_2, 6H_2O$ (A.R.) is dissolved in a litre of water, giving a solution containing 0·1 mg. cobalt per ml. Dilute 50 ml. of this solution to 1 litre, giving 0·005 mg. per ml. Varying amounts of this dilute standard solution are measured by means of N.P.L. standard pipettes into 150 ml. flasks. To each flask is then added distilled water to about 20 ml., 0·5 ml. HCl (1·12) and 6 drops 1 : 1 HNO_3. The solutions are boiled for 2 min., cooled, 2 ml. nitroso-*R*-salt solution and 2 g. sodium acetate added and the cobalt estimated as in (1) above using filter S 53 and 20 mm. cells. The values so obtained (Table I) are found to lie on a straight line.

Table I

ml. standard cobalt solution	mg. cobalt present	Extinction coefficient (k)
0·5	0·0025	0·045
1·0	0·0050	0·088
1·5	0·0075	0·125
2·0	0·0100	0·175
3·0	0·0150	0·263
4·0	0·0200	0·350
5·0	0·0250	0·438
6·0	0·0300	0·525

All readings were taken at 15°.

Experiments were then carried out to test the validity of the graph so obtained after copper and iron had been removed by the methods described. 1·0 and 2·0 ml. of the same standard cobalt solution were used. To the solutions varying amounts of copper and iron were added and these elements then removed, copper by H_2S and by aluminium, and iron by means of zinc oxide. It was found that the extinction coefficients of the final solutions after development of the cobalt complex were respectively 0·088 and $0·175 \pm 0·003$.

Cobalt in grasses. Cobalt estimations were carried out on numerous samples, the quantity found ranging from a mere trace to a figure of 1·4 parts per million. There did not appear to be sufficient copper in any of the samples to affect the result, since treatment with H_2S or aluminium made no difference to the figures.

Recovery of cobalt. This was applied as a further check on the method. To a known weight of a grass there was added a measured volume of standard cobalt solution, and the two ignited together. The cobalt content of the grass was

Table II

Sample no.	Weight taken g.	Added cobalt mg.	Extinction coefficient	Co found mg.	Co calculated mg.	% recovery of Co
5	5	—	0·014	0·0008	—	—
	5	0·0050	0·102	0·0059	0·0058	101·7
12	5	—	0·052	0·0030	—	—
	5	0·0025	0·092	0·0053	0·0055	96·4
	5	0·0050	0·135	0·0078	0·0080	97·5
	5	0·0100	0·215	0·0124	0·0130	95·4
101	10	—	0·065	0·0038	—	—
	10	0·0050	0·150	0·0086	0·0088	97·7

already known, so that the difference between the figures is a measure of the added cobalt. The recovery figures are very satisfactory as can be seen from the results in Table II. Incidentally, on no occasion was any loss of cobalt noted after ignition at low red heat.

It is to be noted that the cobalt present in grass is considerably less than that in the parent soil. For example, two grasses showed cobalt contents of 0·2 and 0·6 p.p.m., whereas the corresponding soils gave figures of 8 and 9 p.p.m. respectively.

SUMMARY

The application of the Pulfrich photometer in place of the tintometer for the estimation of cobalt has been investigated. The instrument is found to be eminently suited for this purpose, since not only does it give rapid and accurate readings, but the colour filters provide a simple means of eliminating the interference of other elements such as iron without having to remove the metal from solution.

Estimation of the cobalt depends on the formation of a complex between it and nitroso-*R*-salt. The method used for the development of the complex in the solution under examination is similar to that given by Kidson *et al.*

The grasses examined showed a very small cobalt content, in the majority of cases less than 1 p.p.m. By means of the photometric method described above, results can be reproduced to an accuracy within 0·1 p.p.m.

Several normal blood samples examined showed no trace of cobalt, nor was this element found in a dried sample of sheep's liver.

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